EP0569438

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

B3

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/32, A01N 63/02
C12Q 1/68, C12N 1/21
C07K 13/00 // (C12N 1/21
C12R 1:07, 1:19)

A1

(11) International Publication Number: WO 92/13954

(43) International Publication Date: 20 August 1992 (20.08.92)

(21) International Application Number: PCT/US92/00040

(22) International Filing Date: 3 January 1992 (03.01.92)

(30) Priority data:
649,562 31 January 1991 (31.01.91) US
813,592 23 December 1991 (23.12.91) US

(71) Applicant: ECOGEN INC. [US/US]; 2005 Cabot Boulevard West, Langhorne, PA 19047-1810 (US).

(72) Inventors: DONOVAN, William, P. ; 36 Calicobush Road, Levittown, PA 19057 (US). RUPAR, Mark, J. ; 42 Sturbridge Drive, Wilmington, DE 19810 (US). SLANEY, Annette, C. ; 4 Dunmoor Court South, Hamilton Square, NJ 08690 (US).

(74) Agents: NADEL, Alan, S.; Panitch Schwarze Jacobs & Nadel, 1601 Market Street - 36th Floor, Philadelphia, PA 19103 (US) et al.

(81) Designated States: AT (European patent), AU, BE (European patent), BR, CA, CH (European patent), CS, DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), MC (European patent), NL (European patent), NO, PL, RU, SE (European patent).

Published
With international search report.

(54) Title: *BACILLUS THURINGIENSIS CRYIIIC(b)* TOXIN GENE AND PROTEIN TOXIC TO COLEOPTERAN INSECTS

(57) Abstract

A *Bacillus thuringiensis* strain isolate, designated EG5144, exhibits insecticidal activity against coleopteran insects, including Colorado potato beetle and insects of the genus *Diabrotica*. A novel toxin gene in *B.t.* strain EG5144 produces an irregularly shaped insecticidal crystal protein of approximately 70 kDa that is toxic to coleopteran insects. The cryIII-type gene (SEQ ID NO:1), designated as the cryIIIC(b) gene, has a nucleotide base sequence illustrated in Figure 1.

NSDOCID: <WO 9213954A1>

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		
ES	Spain	MG	Madagascar		

SDOCID: <WO 9213954A1>

A number of genes encoding crystal proteins have been cloned from several strains of *B.t.* A review of such genes is set forth in H. Höfte et al., *Microbiol. Rev.*, *53*, pp.242-255 (1989). This reference provides a good overview of the genes and proteins obtained from *B.t.* and their uses, adopts a nomenclature and classification scheme for *B.t.* genes and proteins, and has an extensive bibliography.

The *B.t.* crystal protein is toxic in the insect only after ingestion. After ingestion, the alkaline pH and proteolytic enzymes in the insect mid-gut solubilize the crystal allowing the release of the toxic components. These toxic components disrupt the mid-gut cells causing the insect to cease feeding and, eventually, to die. In fact, *B.t.* has proven to be an effective and environmentally safe insecticide in dealing with various insect pests.

As noted by Höfte et al., the majority of insecticidal *B.t.* strains are active against insects of the order Lepidoptera, i.e., caterpillar insects. Other *B.t.* strains are insecticidally active against insects of the order Diptera, i.e., flies and mosquitoes, or against both lepidopteran and dipteran insects. In recent years, a few *B.t.* strains have been reported as producing crystal protein that is toxic to insects of the order Coleoptera, i.e., beetles.

ISDOCID: <WO 9213954A1>

BACILLUS THURINGIENSIS cryIIIC(b) TOXIN GENE AND PROTEIN TOXIC TO COLEOPTERAN INSECTS

Field of the Invention

The present invention relates to an isolated *Bacillus thuringiensis* strain, to its novel toxin encoding gene and to the insecticidal crystal protein toxin made by the gene, as well as to insecticidal compositions containing the protein that are toxic to coleopteran insects.

Background of the Invention

Bacillus thuringiensis (hereinafter "*B.t.*") is a gram-positive soil bacterium that produces crystal proteins during sporulation which are specifically toxic to certain orders and species of insects. Many different strains of *B.t.* have been shown to produce insecticidal crystal proteins. Compositions including *B.t.* strains which produce insecticidal proteins have been commercially available and used as environmentally acceptable insecticides because they are quite toxic to the specific target insect, but are harmless to plants and other non-targeted organisms.

Pseudomonas fluorescens cells harboring the cloned gene were found to be toxic to Colorado potato beetle larva .

PCT International Publication No. WO 91/07481 dated May 30, 1991, of Novo Nordisk A/S, describes *B.t.* mutants that produce high yields of the same insecticidal proteins originally made by the parent strains at lesser yields. Mutants of the coleopteran-toxic *B.t. tenebrionis* strain are disclosed.

A coleopteran-toxic strain, designated *B.t.* var. *san diego*, is reported by C. Herrnstadt et al., *Bio/Technology*, *4*, pp.305-308 (1986), to produce a 64 kDa crystal protein that was toxic to various coleopteran insects: strong toxicity to *Pyrrhalta luteola* (elm leaf beetle); moderate toxicity to *Anthonomus grandis* (boll weevil), *Leptinotarsa decemlineata* (Colorado potato beetle), *Otiorhynchus sulcatus* (black vine weevil), *Tenebrio molitor* (yellow mealworm) and *Haltica tombacina;* and weak toxicity to *Diabrotica undecimpunctata undecimpunctata* (western spotted cucumber beetle).

The DNA sequence of the cloned coleopteran toxin gene of *B.t. san diego* is reported in C. Herrnstadt et al., *Gene*, *57*, pp.37-46 (1987); see also U.S. Patent 4,771,131, issued September 13, 1988, of Herrnstadt et al. The sequence of the toxin gene of *B.t. san diego* is identical to that reported by Sekar et al. (1987) for the cloned coleopteran toxin gene of *B.t. tenebrionis*.

NSDOCID: <WO 9213954A1>

The first isolation of a coleopteran-toxic *B.t.* strain is reported by A. Krieg et al., in *Z.angew.Ent.*, *96*, pp.500-508 (1983); see also A. Krieg et al., *Anz.Schaedlingskde., Pflanzenschutz, Umweltschutz, 57*, pp.145-150 (1984) and U.S. Patent 4,766,203, issued August 23, 1988 of A. Krieg et al. The strain, designated *B.t.* var. *tenebrionis*, is reported to be toxic to larvae of the coleopteran insects *Agelastica alni* (blue alder leaf beetle) and *Leptinotarsa decemlineata* (Colorado potato beetle). *B.t. tenebrionis* makes an insecticidal crystal protein reported to be about 65-70 kilodaltons (kDa) (U.S. Patent 4,766,203; see also K. Bernhard, *FEMS Microbiol.Lett.*, *33*, pp.261-265 (1986)).

V. Sekar et al., *Proc.Natl.Acad.Sci.USA*, *84*, pp.7036-7040 (1987), report the cloning and characterization of the gene for the coleopteran-toxic crystal protein of *B.t. tenebrionis*. The size of the protein, as deduced from the sequence of the gene, was 73 kDa, but the isolated protein contained primarily a 65 kDa component. Höfte et al., *Nucleic Acids Res.*, *15*, p.7183 (1987), also report the DNA sequence for the cloned gene from *B.t. tenebrionis*, and the sequence of the gene is identical to that reported by Sekar et al. (1987).

McPherson et al., *Bio/Technology*, *6*, pp.61-66 (1988), disclose the DNA sequence for the cloned insect control gene from *B.t. tenebrionis*, and the sequence is identical to that r ported by Sekar et al. (1987). *E.coli* cells and

SDOCID: <WO 9213954A1>

gene. The hybrid *B.t.* produces crystal proteins characteristic of those made by *B.t. kurstaki*, as well as those of *B.t. tenebrionis*.

U.S. Patent No. 4,910,016, issued March 20, 1990, of Gaertner et al. (corresponding to EP-A-0 303 379), discloses a novel *B.t.* isolate identified as *B.t.* MT 104 which has insecticidal activity against two orders of insects, Colorado potato beetle (Coleoptera) and cabbage looper (Lepidoptera).

European Patent Application Publication No. 0 318 143, published May 31, 1989, of Lubrizol Genetics, Inc., discloses the cloning, characterization and selective expression of the intact partially modified gene from *B.t. tenebrionis*, and the transfer of the cloned gene into a host microorganism rendering the microorganism able to produce a protein having toxicity to coleopteran insects. Insect bioassay data for *B.t. san diego* reproduced from Herrnstadt et al., *Bio/Technology*, *4*, pp.305-308 (1986) discussed above, is summarized. The summary also includes data for *B.t. tenebrionis* from another source; *B.t. tenebrionis* is reported to exhibit strong toxicity to Colorado potato beetle, moderate toxicity to western corn rootworm (*Diabrotica virgifera*) and weak toxicity to southern corn rootworm (*Diabrotica undecimpunctata*).

European Patent Application Publication No. 0 324 254, published July 19, 1989, of Imperial Chemical Industries PLC, discloses a novel *B.t.* strain identified

SDOCID: <WO 9213954A1>

A. Krieg et al., *J.Appl.Ent.*, *104*, pp.417-424 (1987), report that the strain *B.t. san diego* is identical to the *B.t. tenebrionis* strain, based on various diagnostic tests.

Another new *B.t.* strain, designated EG2158, is reported by W.P. Donovan et al., in *Mol.Gen.Genet.*, *214*, pp.365-372 (1988) and in U.S. Patent No. 5,024,837 issued June 18, 1991, to produce a 73 kDa crystal protein that is insecticidal to coleopteran insects. The toxin-encoding gene from *B.t.* strain EG2158 was cloned and sequenced, and its sequence is identical to that reported by Sekar et al. (1987) for the cloned *B.t. tenebrionis* coleopteran toxin gene. This coleopteran toxin gene is referred to as the *cryIIIA* gene by Höfte et al., *Microbiol.Rev.*, *53*, pp.242-255 (1989).

The Donovan et al. '837 U.S. patent noted above also describes hybrid *B.t.* var. *kurstaki* strains designated EG2424 and EG2421, which are active against both lepidopteran insects and coleopteran insects. The beetle activity of these hybrid strains results from the coleopteran toxin plasmid transferred from *B.t.* strain EG2158 by conjugal plasmid transfer.

U.S. Patent 4,797,279, issued January 10, 1989, of D. Karamata et al. (corresponding to EP-A-0 221 024), discloses a hybrid *B.t.* microorganism containing a plasmid from *B.t.* var. *kurstaki* with a lepidopteran toxin gene and a plasmid from *B.t. tenebrionis* with a coleopteran toxin

:DOCID: <WO 9213954A1>

337 604), discloses a *B.t.* toxin gene obtained from the coleopteran-active *B.t.* strain 43F, and the gene sequence appears identical to the *cryIIIB* gene. *B.t.* strain 43F is reported as being active against Colorado potato beetle and *Leptinotarsa texana*.

European Patent Application No. 0 382 990, published August 22, 1990, of Plant Genetic Systems N.V., discloses two novel *B.t.* strains (btGSI208 and btGSI245) producing respective crystal proteins of 74 and 129 kDa that exhibit insecticidal activity against Colorado potato beetle larvae. The DNA sequence reported for toxin gene producing the 74 kDa protein appears to be identical to that of the *cryIIIB* gene of Sick et al.

PCT International Publication No. WO 90/13651, published November 15, 1990, of Imperial Chemical Industries PLC, discloses novel *B.t.* strains which contain a toxin gene encoding an 81 kDa protein that is stated to be toxic not only to lepidopteran insects but also to coleopteran insects, including Diabrotica.

U.S. Patent No. 5,055,293, issued October 8, 1991, of Aronson et al., discloses the use of *B. laterosporous* for corn rootworm (Diabrotica) insect control.

The various *B.t.* strains described in aforementioned literature are reported to have crystal proteins insecticidally active against coleopteran insects, but none has been demonstrated to have significant, quantifiable toxicity to the larvae and adults of the

as A30 which has insecticidal activity against coleopteran insects, including Colorado potato beetle larvae, corn rootworm larvae and boll weevils.

U.S. Patent No. 4,999,192, issued March 12, 1991, of Payne et al. (corresponding to EP A-0 328 383), discloses a novel *B.t.* microorganism identified as *B.t.* PS40D1 which has insecticidal activity against Colorado potato beetle larvae. *B.t.* strain PS40D1 is identified via serotyping as being serovar 8a8b, *morrisoni*.

U.S. Patent No. 5,006,336, issued April 9, 1991, of Payne et al. (corresponding to EP-A-0 346 114), discloses a novel *B.t.* isolate designated as PS122D3, which is serotyped as serovar 8a8b, *morrisoni* and which exhibits insecticidal activity against Colorado potato beetle larvae.

U.S. Patent No. 4,966,765, issued October 30, 1990, of Payne et al. (corresponding to EP-A-0 330 342), discloses a novel *B.t.* microorganism identified as *B.t.* PS86B1 which has insecticidal activity against the Colorado potato beetle. *B.t.* strain PS86B1 is identified via serotyping as being serovar *tolworthi*.

The nucleotide sequence of a *cryIIIB* gene and its encoded coleopteran-toxic protein is reported by Sick et al., in *Nucleic Acids Res.*, *18*, p.1305 (1990) but the *B.t.* source strain is identified only via serotyping as being subspecies *tolworthi*. U.S. Patent No. 4,966,155, issued February 26, 1991, of Sick et al. (corresponding to EP-A-0

Coleoptera, in particular, Colorado potato beetle and insects of the genus *Diabrotica*.

Still another aspect of the present invention relates to a biologically pure culture of a *B.t.* bacterium deposited with the Agricultural Research Culture Collection, Northern Regional Research Laboratory (NRRL) having Accession No. NRRL B-18655 and being designated as *B.t.* strain EG5144 and a biologically pure culture of a second bacterium deposited with the NRRL having Accession No. NRRL B-18920 and being designated as *B.t.* strain EG5145. *B.t.* strain EG5144 is a wild-type *B.t.* strain that carries the *cryIIIC(b)* gene (SEQ ID NO:1) and produces the insecticidal CryIIIC(b) protein (SEQ ID NO:2). *B.t.* strain EG5145 is also a wild-type *B.t.* strain, whose characteristics are similar to those of *B.t.* strain EG5144 described in more detail below. Biologically pure cultures of other B.t. bacteria carrying the *cryIIIC(b)* gene (SEQ ID NO:1) are also within the scope of this invention.

Yet another aspect of this invention relates to insecticidal compositions containing, in combination with an agriculturally acceptable carrier, either the CryIIIC(b) protein (SEQ ID NO:2) or fermentation cultures of a *B.t.* strain which has produced the CryIIIC(b) protein.

The invention also includes a method of controlling coleopteran insects by applying to a host plant for such

insect genus *Diabrotica* (corn rootworm), which includes the western corn rootworm (*Diabrotica virgifera virgifera*), the southern corn rootworm (*Diabrotica undecimpunctata howardi*) and the northern corn rootworm (*Diabrotica barberi*).

The *B.t.* strain of the present invention contains a novel toxin gene that expresses protein toxin having quantifiable insecticidal activity against the *Diabrotica* insects, among other coleopteran insects.

Summary of the Invention

One aspect of the present invention relates to a purified and isolated coleopteran toxin gene having a nucleotide base sequence coding for the amino acid sequence illustrated in Figure 1 and hereinafter designated as the *cryIIIC(b)* gene (SEQ ID NO:1). The *cryIIIC(b)* gene (SEQ ID NO:1) has a coding region extending from nucleotide bases 144 to 2099 shown in Figure 1.

Another aspect of the present invention relates to the insecticidal protein produced by the *cryIIIC(b) gene*. The CryIIIC(b) protein (SEQ ID NO:2) has the amino acid sequence, as deduced from the nucleotide sequence of the *cryIIIC(b)* gene (SEQ ID NO:1) from nucleotide bases 144 to 2099 that is shown in Figure 1. The protein exhibits insecticidal activity against insects of the order

left of Figure 2 indicate the approximate sizes, in megadaltons (MDa), of the plasmids of *B.t.* strain EG5144.

Figure 3 is a photograph of an autoradiogram made by transferring size fractionated DNA fragments from an agarose gel to a nitrocellulose filter, hybridizing the filter with a radioactively labeled 2.4 kilobases (kb) *cryIIIB* probe, and exposing the filter to X-ray film. The agarose gel contained size fractionated total DNA fragments from *B.t.* strains EG2158, EG5144, EG2838 and EG4961, that had been obtained in separate digestions with the restriction enzymes *Ssp*I, *Hin*dIII and *Eco*RI. The numbers to the left of Figure 3 indicate the sizes, in kb, of *B.t.* strain EG5144 restriction fragments that hybridized to the *cryIIIB* probe. The lane labeled "stnd" is a size standard.

Figure 4 is a photograph of a Coomassie stained sodium dodecyl sulfate ("SDS") polyacrylamide gel showing crystal proteins solubilized from *B.t.* strains EG5144 (lane 1), EG4961 (lane 2), EG2158 (lane 3) and EG2838 (lane 4). The numbers to the left of Figure 4 indicate the approximate sizes in kDa of the crystal proteins produced by *B.t.* strain EG5144. Lane 5 contains protein molecular size standards.

Figure 5 shows a restriction map of plasmid pEG271. The location and orientation of the *cryIIIC(b)* gene (SEQ ID NO:1) is indicated by the arrow. Plasmid pEG271 is functional in *Escherichia coli* (*E.coli*), since it contains

ISDOCID: <WO 9213954A1>

insects an insecticidally effective amount of the CryIIIC(b) protein (SEQ ID NO:2) or of a fermentation culture of a *B.t.* strain that has made the CryIIIC(b) protein. The method is applicable to a variety of coleopteran insects, such as the Colorado potato beetle, Japanese beetle larvae (white grubs), Mexican bean beetle and corn rootworm.

Still another aspect of the present invention relates to a recombinant plasmid containing the *cryIIIC(b)* gene (SEQ ID NO:1), a biologically pure culture of a bacterium transformed with such recombinant plasmid, the bacterium preferably being *B.t.*, such as *B.t.* strain EG7237 described in Example 6, as well as a plant transformed with the *cryIIIC(b)* gene.

Brief Description of the Drawings

Figure 1 comprises Figures 1-1 through 1-3 and shows the nucleotide base sequence of the *cryIIIC(b)* gene (SEQ ID NO:1) and the deduced amino acid sequence of the CryIIIC(b) protein (SEQ ID NO:2). The putative ribosome binding site (RBS) is indicated. Restriction sites for *SspI* and *Hin*dIII are also indicated.

Figure 2 is a photograph of an ethidium bromide stained agarose gel containing size fractionated native plasmids of *B.t.* strains EG5144 (lane 1), EG4961 (lane 2), EG2838 (lane 3) and EG2158 (lane 4). The numbers to the

DOCID: <WO 9213954A1>

are described at length in Examples 1-7. The utility of *B.t.* strain EG5144 and of the CryIIIC(b) crystal protein (SEQ ID NO:2) in insecticidal compositions and methods is also illustrated in Examples 8-11.

The *cryIII*-type gene of this invention, the *cryIIIC(b)* gene (SEQ ID NO:1), has the nucleotide bas sequence shown in Figure 1. The coding region of the *cryIIIC(b)* gene (SEQ ID NO:1) extends from nucleotide base position 144 to position 2099 shown in Figure 1.

A comparison of the nucleotide base sequence of the *cryIIIC(b)* gene coding region with the corresponding coding region of the prior art *cryIIIA* gene indicates significant differences between the two genes. The *cryIIIC(b)* gene (SEQ ID NO:1) is only 76% homologous (positionally identical) with the *cryIIIA* gene.

A comparison of the nucleotide base sequence of the *cryIIIC(b)* gene coding region with the corresponding coding region of the *cryIIIB* gene obtained from recently discovered *B.t.* strain EG2838 (NRRL Accession No. B-18603) indicates that the *cryIIIC(b)* gene (SEQ ID NO:1) is 96% homologous (positionally identical) with the *cryIIIB* gene.

The CryIII-type protein of this invention, the CryIIIC(b) protein, that is encoded by the *cryIIIC(b)* gene (SEQ ID NO:1), has the amino acid sequence (SEQ ID NO:2) shown in Figure 1. In this disclosure, references to the CryIIIC(b) "protein" are synonymous with its description as a "crystal protein", "protein toxin", "insecticidal

E.coli plasmid pUC18 (Ap^r), indicated by the segment marked pUC18. The abbreviations for the restriction endonuclease cleavage sites are as follows: Ba=*Bam*HI; Bg=*Bgl*II; H=*Hin*dIII; R=*Eco*RI; S=*Sph*I; and X=*Xba*I. A one kilobase scale marker is also illustrated.

Figure 6, aligned with and based on the same scal as Figure 5, shows a restriction map of plasmid pEG272. The location and orientation of the *cryIIIC(b)* gene (SEQ ID NO:1) is indicated by the arrow shown in Figure 5. Plasmid pEG272 is derived from plasmid pEG271 (Figure 5) and contains the *Bacillus* plasmid pNN101 (Cm^r Tc^r), indicated by the segment marked pNN101 and is incorporated into the *Sph*I site of pEG271; this plasmid is functional in *B.t.* Abbreviations are the same as those for Figure 5.

Figure 7 is a photograph of a Coomassie stained SDS-polyacrylamide gel. The gel shows protein bands synthesized by *B.t.* strain EG5144 (lane 1) and by recombinant *B.t.* strain EG7237 containing pEG272 (lane 3). Lane 2 contains a protein size standard and the numbers on either side of lanes 1 and 3 indicate approximate sizes, in kDa, of the crystal proteins produced by these strains.

Detailed Description of the Preferred Embodiments

The isolation and purification of the *cryIIIC(b)* gene (SEQ ID NO:1) and the coleopteran-toxic CryIIIC(b) crystal protein (SEQ ID NO:2) and the characterization of the new *B.t.* strain EG5144 which produces the CryIIIC(b) protein

DOCID: <WO 9213954A1>

e.g., truncated versions, of the *cryIIIC(b)* gene (SEQ ID NO:1) that yield a protein with insecticidal properties essentially the same as those of the CryIIIC(b) protein (SEQ ID NO:2).

The *cryIIIC(b)* gene (SEQ ID NO:1) is also useful as a DNA hybridization probe, for discovering similar or closely related *cryIII*-type genes in other *B.t.* strains. The *cryIIIC(b)* gene (SEQ ID NO:1), or portions or derivatives thereof, can be labeled for use as a hybridization probe, e.g., with a radioactive label, using conventional procedures. The labeled DNA hybridization probe may then be used in the manner described in the Examples.

The *cryIIIC(b)* gene (SEQ ID NO:1) and the corresponding insecticidal CryIIIC(b) protein (SEQ ID NO:2) were first identified in *B.t.* strain EG5144, a novel *B.t.* isolate. The characteristics of *B.t.* strain EG5144 are more fully described in the Examples. Comparison of the plasmid arrays and other strain characteristics of *B.t.* strain EG5144 with those of the recently discovered *B.t.* strains EG2838 and EG4961 and those of the prior art *B.t.* strain EG2158 and *B.t.* var. *tenebrionis* (or the equivalent, *B.t.* var. *san diego*) demonstrates that each of these coleopteran-toxic *B.t.* strains is distinctly different. The plasmid array of *B.t.* strain EG5145, another wild-type strain isolated along with *B.t.* strain EG5144, is similar to that of *B.t.* strain EG5144, and *B.t.*

protein" or the like, unless the context indicates otherwise. The size of the CryIIIC(b) protein (SEQ ID NO:2), as deduced from the DNA sequence of the *cryIIIC(b)* gene (SEQ ID NO:1), is 74,265 Daltons (Da).

The size of the CryIIIB protein, as deduced from the sequence of the *cryIIIB* gene, is 74,237 Da. The prior art CryIIIA protein, encoded by the *cryIIIA* gene, has a deduced size of 73,116 Da.

Despite the apparent size similarity, comparison of the amino acid sequence of the CryIIIC(b) protein (SEQ ID NO:2) with that of the prior art CryIIIA protein shows significant differences between the two. The CryIIIC(b) protein (SEQ ID NO:2) is only 68% homologous (positionally identical amino acids) with the CryIIIA protein. The CryIIIC(b) protein (SEQ ID NO:2) is 95% homolgous with the CryIIIB protein. Nevertheless, despite the apparent homology of the CryIIIC(b) and CryIIIB proteins, the CryIIIC(b) protein (SEQ ID NO:2) has been shown to be a different protein than the CryIIIB protein, based on its significantly improved insecticidal activity compared to the CryIIIB protein with respect to insects of the order Coleoptera and in particular, insects of the genus *Diabrotica*. The CryIIIC(b) protein (SEQ ID NO:2), unlik the CryIIIB protein, exhibits quantifiable insecticidal activity against corn rootworm larvae.

The present invention is intended to cover mutants and recombinant or genetically engineered derivatives,

is formed along with spores. The *B.t.* fermentation culture is then typically harvested by centrifugation, filtration or the like to separate fermentation culture solids, containing the CryIIIC(b) crystal protein, from the aqueous broth portion of the culture.

The *B.t.* strains exemplified in this disclosure are sporulating varieties (spore forming or sporogenous strains) but the *cryIIIC(b)* gene (SEQ ID NO:1) also has utility in asporogenous *Bacillus* strains, i.e., strains that produced the crystal protein without production of spores. It should be understood that references to "fermentation cultures" of *B.t.* strains (containing the *cryIIIC(b)* gene (SEQ ID NO:1)) in this disclosure are intended to cover sporulated *B.t.* cultures, i.e., *B.t.* cultures containing the CryIIIC(b) crystal protein and spores, and sporogenous *Bacillus* strains that have produced crystal protein during the vegetative stage, as well as asporogenous *Bacillus* strains containing the *cryIIIC(b)* gene (SEQ ID NO:1) in which the culture has reached the growth stage where crystal protein is actually produced.

The separated fermentation solids are primarily CryIIIC(b) crystal protein (SEQ ID NO:2) and *B.t.* spores, along with some cell debris, some intact cells, and residual fermentation medium solids. If desired, the crystal protein may be separated from the other recovered solids via conventional methods, e.g., sucrose density

strain EG5145 exhibits the same insecticidal activity against coleopteran insects, e.g., Japanese beetle larvae, as that of *B.t.* strain EG5144 (see Example 11).

The *cryIIIC(b)* gene (SEQ ID NO:1) may be introduced into a variety of microorganism hosts, using procedures well known to those skilled in the art for transforming suitable hosts under conditions which allow for stable maintenance and expression of the cloned *cryIIIC(b)* gene. Suitable hosts that allow the *cryIIIC(b)* gene (SEQ ID NO:1) to be expressed and the CryIIIC(b) protein (SEQ ID NO:2) to be produced include *Bacillus thuringiensis* and other *Bacillus* species such as *B. subtilis* or *B. megaterium*. It should be evident that genetically altered or engineered microorganisms containing the *cryIIIC(b)* gene (SEQ ID NO:1) can also contain other toxin genes present in the same microorganism and that these genes could concurrently produce insecticidal crystal proteins different from the CryIIIC(b) protein.

The *Bacillus* strains described in this disclosure may be cultured using conventional growth media and standard fermentation techniques. The *B.t.* strains harboring the *cryIIIC(b)* gene (SEQ ID NO:1) may be fermented, as described in the Examples, until the cultured *B.t.* cells reach the stage of their growth cycle when CryIIIC(b) crystal protein (SEQ ID NO:2) is formed. For sporogenous *B.t.* strains, fermentation is typically continued through the sporulation stage when the CryIIIC(b) crystal protein

DOCID: <WO 9213954A1>

microorganism host carrying the *cryIIIC(b)* gene (SEQ ID NO:1) and capable of producing the CryIIIC(b) protein. Preferred *Bacillus* hosts include *B.t.* strain EG5144 and genetically improved *B.t.* strains derived from *B.t.* strain EG5144. The latter *B.t.* strains may be obtained via plasmid curing and/or conjugation techniques and contain the native *cryIIIC(b)* gene-containing plasmid from *B.t.* strain EG5144. Genetically engineered or transformed *B.t.* strains or other host microorganisms containing a recombinant plasmid that expresses the cloned *cryIIIC(b)* gene (SEQ ID NO:1), obtained by recombinant DNA procedures, may also be used.

An example of such transformants is *B.t.* strain EG7237, which contains the cloned *cryIIIC(b)* gene (SEQ ID NO:1) on a recombinant plasmid.

The recovered fermentation solids contain primarily the crystal protein and (if a sporulating *B.t.* host is employed) spores; cell debris and residual fermentation medium solids may also be present. The recovered fermentation solids containing the CryIIIC(b) protein may be dried, if desired, prior to incorporation in the insecticidal formulation.

The formulations or compositions of this invention containing the insecticidal CryIIIC(b) protein (SEQ ID NO:2) as the active component are applied at an insecticidally effective amount which will vary depending on such factors as, for example, the specific coleopteran

NSDOCID: <WO 9213954A1>

gradient fractionation. Highly purified CryIIIC(b) protein (SEQ ID NO:2) may be obtained by solubilizing the recovered crystal protein and then precipitating the protein from solution.

The CryIIIC(b) protein (SEQ ID NO:2), as noted earlier, is a potent insecticidal compound against coleopteran insects, such as the Colorado potato beetle, Japanese beetle larvae (white grubs), Mexican bean beetle and the like. The CryIIIC(b) protein (SEQ ID NO:2), in contrast to the CryIIIA and CryIIIB proteins, exhibits measurable insecticidal activity against *Diabrotica* insects, e.g., corn rootworms, which have been relatively unaffected by other coleopteran-toxic *B.t.* crystal proteins. The CryIIIC(b) protein (SEQ ID NO:2) may be utilized as the active ingredient in insecticidal formulations useful for the control of coleopteran insects such as those mentioned above. Such insecticidal formulations or compositions typically contain agriculturally acceptable carriers or adjuvants in addition to the active ingredient and are prepared and used in a manner well known to those skilled in the art.

The CryIIIC(b) protein (SEQ ID NO:2) may be employed in insecticidal formulations in isolated or purified form, e.g., as the crystal protein itself. Alternatively, the CryIIIC(b) protein (SEQ ID NO:2) may be present in the recovered fermentation solids, obtained from culturing of a *Bacillus* strain, e.g., *Bacillus thuringiensis*, or other

DOCID: <WO 9213954A1>

insect, typically onto the foliage of the plant or crop to be protected, by conventional methods, preferably by spraying. Other application techniques, e.g., dusting, sprinkling, soaking, soil injection, seed coating, seedling coating or spraying, or the like, are also feasible and may be required for insects that cause root or stalk infestation. These application procedures are well known in the art.

The *cryIIIC(b)* gene (SEQ ID NO:1) or its functional equivalent, hereinafter sometimes referred to as the "toxin gene," can be introduced into a wide variety of microorganism hosts. Expression of the *cryIIIC(b)* gene (SEQ ID NO:1) results in the production of insecticidal CryIIIC(b) crystal protein toxin (SEQ ID NO:2). Suitable hosts include *B.t.* and other species of *Bacillus*, such as *B. subtilis* or *B. megaterium*, for example. Plant-colonizing or root-colonizing microorganisms may also be employed as the host for the *cryIIIC(b)* gene (SEQ ID NO:1). Various procedures well known to those skilled in the art are available for introducing the *cryIIIC(b)* gene (SEQ ID NO:1) into the microorganism host under conditions which allow for stable maintenance and expression of the gene in the resulting transformants.

The transformants, i.e., host microorganisms that harbor a cloned gene in a recombinant plasmid, can be isolated in accordance with conventional methods, usually employing a selection technique, which allows growth of

SUBSTITUTE SHEET (RULE 26)

insects to be controlled, the specific plant or crop to be treated and the method of applying the insecticidally active compositions. An insecticidally effective amount of the insecticide formulation is employed in the insect control method of this invention.

The insecticide compositions are made by formulating the insecticidally active component with the desired agriculturally acceptable carrier. The formulated compositions may be in the form of a dust or granular material, or a suspension in oil (vegetable or mineral) or water or oil/water emulsions, or as a wettable powder, or in combination with any other carrier material suitable for agricultural application. Suitable agricultural carriers can be solid or liquid and are well known in the art. The term "agriculturally acceptable carrier" covers all adjuvants, e.g., inert components, dispersants, surfactants, tackifiers, binders, etc. that are ordinarily used in insecticide formulation technology; these are well known to those skilled in insecticide formulation.

The formulations containing the CryIIIC(b) protein (SEQ ID NO:2) and one or more solid or liquid adjuvants are prepared in known manners, e.g., by homogeneously mixing, blending and/or grinding the insecticidally active CryIIIC(b) protein component with suitable adjuvants using conventional formulation techniques.

The insecticidal compositions of this invention ar applied to the environment of the target coleopteran

introducing DNA into plant tissue is disclosed in European Patent Application Publication No. 0 289 479, published November 2, 1988, of Monsanto Company.

DNA containing the *cryIIIC(b)* gene (SEQ ID NO:1) or a modified *cryIIIC(b)* gene capable of producing the CryIIIC(b) protein (SEQ ID NO:2) may be delivered into the plant cells or tissues directly by infectious plasmids, such as Ti, the plasmid from *Agrobacterium tumefaciens*, viruses or microorganisms like *A. tumefaciens*, by the use of lysosomes or liposomes, by microinjection by mechanical methods and by other techniques familiar to those skilled in plant genetic engineering.

Variations may be made in the *cryIIIC(b)* gene nucleotide base sequence (SEQ ID NO:1), since the various amino acids forming the protein encoded by the gene usually may be determined by more than one codon, as is well known to those skilled in the art. Moreover, there may be some variations or truncation in the coding regions of the *cryIIIC(b)* nucleotide base sequence which allow expression of the gene and production of functionally equivalent forms of the CryIIIC(b) insecticidal protein. These variations which can be determined without undue experimentation by those of ordinary skill in the art with reference to the present specification are to be considered within the scope of the appended claims, since they are fully equivalent to the specifically claimed subject matter.

SDOCID: <WO 9213954A1>

only those host microorganisms that contain a recombinant plasmid. The transformants then can be tested for insecticidal activity. Again, these techniques are standard procedures.

Characteristics of particular interest in selecting a host cell for purposes of production include ease of introducing the gene into the host, availability of expression systems, efficiency of expression, stability of the CryIIIC(b) insecticidal protein in the host, and the presence of auxiliary genetic capabilities. The cellular host containing the insecticidal *cryIIIC(b)* gene (SEQ ID NO:1) may be grown in any convenient nutrient medium, where expression of the *cryIIIC(b)* gene is obtained and CryIIIC(b) protein (SEQ ID NO:2) produced, typically to sporulation. The sporulated cells containing the crystal protein may then be harvested in accordance with conventional methods, e.g., centrifugation or filtration.

The *cryIIIC(b)* gene (SEQ ID NO:1) may also be incorporated into a plant which is capable of expressing the gene and producing CryIIIC(b) protein (SEQ ID NO:2), rendering the plant more resistant to insect attack. Genetic engineering of plants with the *cryIIIC(b)* gene (SEQ ID NO:1) may be accomplished by introducing the desired DNA containing the gene into plant tissues or cells, using DNA molecules of a variety of forms and origins that are well known to those skilled in plant genetic engineering. An example of a technique for

A modified treatment procedure was developed for use with *B.t.* colonies utilized in the colony hybridization procedure, since standard techniques applicable to *E.coli* were found to be unworkable with *B.t.* In the treatment described above, special conditions were required to assure that the *B.t.* colonies were in a vegetative state of growth, making them susceptible to lysis with NaOH. Accordingly, after a portion of each colony was transferred to the nitrocellulose filter, the filter was placed colony side up on an agar medium containing 0.5% (w/v) glucose. The transferred colonies were then allowed to grow on the agar-glucose medium for 5 hours at 30°C. Use of 0.5% glucose in the agar medium and the 5-hour, 30°C growth cycle were critical for assuring that the *B.t.* colonies were in a vegetative state and thus susceptible to lysis.

A cloned coleopteran toxin gene was used as a specific probe to find other novel and rare coleopteran-toxic strains of *B.t.* from crop dust samples. A 2.9 kb *Hin*dIII DNA restriction fragment containing the *cryIIIA* gene, formerly known as the *cryC* gene of *B.t.* strain EG2158, described in Donovan et al., *Mol.Gen.Genet., 214,* pp.365-372 (1988), was used as a probe in colony hybridization procedures.

The 2.9 kb *Hin*dIII *cryIIIA* DNA fragment, containing the entire *cryIIIA* gene, was radioactively labeled with [alpha-P^{32}]-dATP and Klenow enzyme, by standard methods.

ISDOCID: <WO 9213954A1>

The present invention will now be described in more detail with reference to the following specific, non-limiting examples. The examples relate to work which was actually done based on techniques generally known in the art and using commercially available equipment.

The novel *B.t.* strain EG5144 was isolated following the procedure described in Example 1. The procedures described in Example 1 were also used to isolate the novel *B.t.* strain EG5145.

Example 1

Isolation of *B.t.* Strains EG5144 and EG5145

Crop dust samples were obtained from various sources throughout the U.S. and abroad, typically grain storage facilities. The crop dust samples were treated by suspending the crop dust in an aqueous buffer and heating the suspension at 60°C for 30 min. to enrich for heat resistant spore forming *Bacillus*-type bacteria such as *B.t.* The treated dust suspensions were diluted in aqueous buffer, and the dilutions were spread on agar plates to allow each individual bacterium from the crop dust to grow into a colony on the surface of the agar plate. After growth, a portion of each colony was transferred from the agar plate to a nitrocellulose filter. The filter was treated with NaOH to lyse the colonies and to fix the DNA from each colony onto the filter.

DOCID: <WO 9213954A1>

fragment contains the 3'-truncated *cryIIIA* gene. When the 2.0 kb fragment was used in repeated colony hybridization experiments, it did not hybridize to *cryI* gene-containing *B.t.* colonies.

Approximately 48,000 *Bacillus*-type colonies from crop dust samples from various locations were probed with the radioactively labeled 2.0 kb *Hin*dIII-*Xba*I *cryIIIA* probe. Only one novel *B.t.* strain from an Illinois crop dust sample was discovered that specifically hybridized to the *cryIIIA* probe. That novel strain was designated *B.t.* strain EG2838, which has been deposited with the NRRL under Accession No. NRRL B-18603.

Subsequently, approximately 50,000 additional *Bacillus*-type colonies from crop dust samples were also screened with the radioactively labeled 2.0 kb *Hin*dIII-*Xba*I *cryIIIA* probe, but without success in identifying any other strains containing novel *cryIII*-type genes.

B.t. strain EG2838 was found to be insecticidally active against coleopteran insects, notably, the Colorado potato beetle. *B.t.* strain EG2838 did not have substantial insecticidal activity with respect to the southern corn rootworm. A gene, designated the *cryIIIB* gene, was isolated from *B.t.* strain EG2838, and its nucleotide base sequence determined. The *cryIIIB* gene encoded a crystal protein, designated the CryIIIB protein, containing 651 amino acids having a deduced size of 74,237 Daltons. The size of the prior art CryIIIA protein had

VSDOCID: <WO 9213954A1>

The nitrocellulose filters containing the DNA from each lysed colony were incubated at 65°C for 16 hours in a buffered solution that contained the radioactively labeled 2.9 kb *Hin*dIII *cryIIIA* DNA probe to hybridize the DNA from the colonies with the DNA from the radioactively labeled *cryIIIA* probe. The 65°C hybridization temperature was used to assure that the *cryIIIA* DNA probe would hybridize only to DNA from colonies that contained a gene that was similar to the *cryIIIA* DNA probe.

The 2.9 kb *cryIIIA* probe hybridized to many *B.t.* colonies from various samples of crop dust. Examination of these colonies revealed, unexpectedly, that they did not contain any *cryIII*-type genes. These colonies did contain *cryI*-type genes. The *cryI*-type genes encode lepidopteran-toxic, coleopteran-nontoxic crystal proteins with molecular masses of approximately 130 kDa. Computer-assisted comparisons of the sequence of the *cryIIIA* gene with the sequence of several *cryI*-type genes revealed that the 3'-end of the *cryIIIA* gene was partially homologous with portion of the *cryI*-type genes. This finding supported the belief that the 3'-end of the *cryIIIA* gene was causing the 2.9 kb *cryIIIA* probe to hybridize to *B.t.* colonies containing *cryI*-type genes.

To correct this problem, the 2.9 kb *Hin*dIII *cryIIIA* probe was digested with the enzyme *Xba*I and a 2.0 kb *Hin*dIII-*Xba*I fragment was purified that contained the *cryIIIA* gene minus its 3'-end. The 2.0 kb *Hin*dIII-*Xba*I

with similarly-processed total DNA from other *B.t.* strains containing *cryIII*-type toxin genes, to demonstrate that *B.t.* strain EG5144 contains a unique coleopteran-active toxin gene. In addition, *B.t.* strain EG5144 was evaluated further by characterizing the crystal proteins it produces and by measuring the insecticidal activity associated with *B.t.* strain EG5144 and its crystal proteins. Examples 2 through 7 are directed to the procedures for characterizing *B.t.* strain EG5144 and its unique *cryIII*-type gene, and Examples 8 through 11 are directed to the insecticidal activity of *B.t.* strain EG5144 and of *B.t.* strain EG7237, containing the *cryIIIC(b)* gene (SEQ ID NO:1) of this invention.

Example 2

Evaluation of the Flagellar Serotype of *B.t.* Strain EG5144

Flagellar serotyping studies were carried out with *B.t.* strain EG5144, using an antibody mediated cell agglutinization assay (Craigie et al., *J.Immunol.*, *21*, pp.417-511 (1936)). Flagellar antibody reagents were prepared using purified flagella from *B.t.* var. *kurstaki*, *morrisoni* and *tolworthi* type-strains and from the novel coleopteran-active *B.t.* strain EG4961.

The study included formalin-fixed vegetative cells of *B.t.* strain EG5144 and of cells of other coleopteran-active *B.t.* strains and of several common *B.t.* type-

previously been deduced to be 73,116 Daltons (644 amino acids). The *cryIIIB* gene is 75% homologous with the *cryIIIA* gene, and the CryIIIB protein is 68% homologous with the CryIIIA protein.

Thousands of *Bacillus*-type colonies from numerous crop dust samples from various locations from around the world were screened with a *cryIIIB* probe obtained from *B.t.* strain EG2838. The *cryIIIB* probe was radioactively labeled using the procedure set forth above with respect to the radioactively labeled *cryIIIA* probe. The radioactively labeled *cryIIIB* probe consisted of a 2.4 kb *Ssp*I restriction fragment of DNA from *B.t.* strain EG2838. The fragment contains the complete protein coding region for the coleopteran toxin *cryIIIB* gene of *B.t.* strain EG2838. Ultimately, the *B.t.* strains of the present invention, designated *B.t.* strains EG5144 and EG5145, were isolated from a crop dust sample via *B.t.* colonies that specifically hybridized to the *cryIIIB* probe.

To characterize *B.t.* strain EG5144, several studies were conducted. One series of studies was performed to characterize its flagellar serotype. Additional studies were conducted to determine the sizes of the native plasmids in *B.t.* strain EG5144 and to ascertain which plasmids contained genes that encoded coleopteran-active insecticidal crystal proteins. DNA blot analysis was thereafter performed using size fractionated total DNA restriction fragments from *B.t.* strain EG5144, compared

)OCID: <WO 9213954A1>

Other *B.t.* flagellar type-strains:

B.t. var. *kurstaki* (HD-1)	+	-	-	-
B.t. var. *morrisoni* (HD-12)	-	+	-	-
B.t. var. *tolworthi* (HD-13)	-	-	+	-

The results in Table 1 show that cells of *B.t.* strain EG5144 gave a negative reaction with *B.t.* type-strain *kurstaki*, *morrisoni* and *tolworthi* flagella antibody reagents. *B.t.* strain EG5144 cells also gave a negative reaction with flagellar reagent from *B.t.* strain EG4961, a novel coleopteran-active strain that has been discovered to exhibit Diabrotica toxicity.

These results indicate that *B.t.* strain EG5144 is not a *kurstaki*, *morrisoni* or *tolworthi*-type *B.t.* strain. Furthermore, the flagellar serotype of *B.t.* strain EG5144, which is yet not known, is apparently different from that of *B.t.* strain EG4961, which has been serotyped as serovar *kumamotoensis* (serotype 18). Both *B.t.* strain EG5144 and *B.t.* strain EG4961 appear to have flagellar serotypes that are different from those of other coleopteran-toxic *B.t.* strains reported in the literature.

Example 3

Size Fractionation and *cryIIIB* Probing of Native Plasmids of EG5144

B.t. strains may be characterized by fractionating their plasmids according to size by the well-known procedure of agarose gel el ctrophoresis. This procedure

ISDOCID: <WO 9213954A1>

strains, each of which were scored for flagellar antibody mediated cell agglutinization.

The other coleopteran-active *B.t.* strains included *B.t.* var. *tenebrionis*, *B.t.* var. *san diego*, *B.t.* strain EG2158 (all containing the *cryIIIA* gene); *B.t.* strain EG2838 (containing the *cryIIIB* gene); and *B.t.* strain EG4961 (containing a novel coleopteran toxin-encoding gene designated as the *cryIIIC(a)* gene).

The *B.t.* flagellar type-strains were *B.t.* var. *kurstaki* (HD-1, serotype 3ab), *B.t.* var. *morrisoni* (HD-12, serotype 8ab) and *B.t.* var. *tolworthi* (HD-13, serotype 9).

Results of this study are shown in Table 1; "+" indicates that a cross-reaction occurred and "-" indicates that no cross-reaction occurred.

Table 1

	Flagellar Antibody Reagent			
Cells	*kurstaki*	*morrisoni*	*tolworthi*	*EG4961*
B.t. strain EG5144	-	-	-	-
B.t. var. *tenebrionis*	-	+	-	-
B.t. var. *san diego*	-	+	-	-
B.t. strain EG2158	-	+	-	-
B.t. strain EG2838	-	-	+	-
B.t. strain EG4961	-	-	-	+

IDOCID: <WO 9213954A1>

from the sizes of the native plasmids of *B.t.* strains EG2158, EG2838 and EG4961. *B.t.* strain EG5144 is therefore distinct from the other coleopteran-toxic *B.t.* strains EG2158, EG2838 and EG4961, based on these plasmid array studies and on the serotyping studies described in Example 2. Likewise, *B.t.* strain EG5145 appears distinct from the coleopteran-toxic *B.t.* strains noted above based on plasmid array studies.

The plasmids shown in Figure 2 were transferred by blotting from the agarose gel to a nitrocellulose filter using the blot techniques of Southern, *J.Molec.Biol.*, *98*, pp.503-517 (1975), and the filter was hybridized as described above with the radioactively labeled 2.4 kb *cryIIIB* DNA probe. After hybridization, the filter was exposed to X-ray film. Examination of the X-ray film confirmed that the *cryIIIB* probe specifically hybridized to the 92 MDa plasmid of *B.t.* strain EG5144. This result demonstrates that the 92 MDa plasmid of *B.t.* strain EG5144 contains a DNA sequence that is at least partly homologous to the *cryIIIB* gene and confirms that the 92 MDa plasmid contains a *cryIII*-type gene. The X-ray film also showed that the *cryIIIB* probe hybridized, as expected, to the 95 MDa plasmid of *B.t.* strain EG4961 and to the 100 MDa plasmid of *B.t.* strain EG2838, and to the 88 MDa plasmid of *B.t.* strain EG2158. The 88 MDa plasmid of *B.t.* strain EG2158 has been previously shown to contain the coleopteran-toxin *cryIIIA* gene (s e Donovan et al.,

involves lysing *B.t.* cells with lysozyme and SDS, electrophoresing plasmids from the lysate through an agarose gel and staining the gel with ethidium bromide to visualize the plasmids. Larger plasmids, which move more slowly through the gel, appear at the top of the gel and smaller plasmids appear toward the bottom of the gel.

The agarose gel in Figure 2 shows that *B.t.* strain EG5144 contains native plasmids of approximately 145, 92, 12, 10 and 5.5 MDa, as indicated by the white horizontal bands. Plasmid sizes were estimated by comparison to plasmids of known sizes (not shown). Although not shown on Figure 2, *B.t.* strain EG5145 contains native plasmids of approximately 145, 92, 12 and 5.5 MDa. The cryptic 10 MDa plasmid found in *B.t.* strain EG5144 is not present in *B.t.* strain EG5145.

Figure 2 further shows that the coleopteran-toxic *B.t.* strain EG4961 contains native plasmids of about 150, 95, 70, 50, 5 and 1.5 MDa and that the coleopteran-toxic *B.t.* strain EG2838 contains native plasmids of about 100, 90 and 37 MDa. Figure 2 also shows that the coleopteran-toxic *B.t.* strain EG2158 contains native plasmids of about 150, 105, 88, 72, and 35 MDa. Some of the plasmids, such as the 150 and 1.5 MDa plasmids of *B.t.* strain EG4961 and the 150 MDa plasmid of *B.t.* strain EG2158, may not be visible in the photograph, although they are visible in the actual gel. Figure 2 demonstrates that the sizes of the nativ plasmids of *B.t.* strain EG5144 are different

DOCID: <WO 9213954A1>

Mol.Gen.Genet., *214*, pp.365-372 (1988)). The inventors have previously determined that the 100 MDa plasmid of *B.t.* strain EG2838 contains the coleopteran toxin *cryIIIB* gene and that the 95 MDa plasmid of *B.t.* strain EG4961 contains the novel coleopteran toxin *cryIIIC(a)* gene.

Example 4

Blot Analysis of DNA from *B.t.* Strains EG5144 and EG5145

Both chromosomal and plasmid DNA (total DNA) from *B.t.* strain EG5144 were extracted and digested with separate restriction enzymes, *Ssp*I, *Hin*dIII and *Eco*RI. The digested DNA was size fractionated by electrophor sis through an agarose g , and the fragments were then visualized by staining with ethidium bromide. For comparison, total DNA from the coleopteran-toxic *B.t.* strains EG2158, EG2838 and EG4961 was processed in an identical manner. Examination of the resultant stained agarose gel showed that restriction digestions of total DNA from these *B.t.* strains with each of *Ssp*I, *Hin*dIII and *Eco*RI yield hundreds of DNA fragments of various sizes.

The size fractionated DNA restriction fragments were transferred by blotting from the agarose gel to a nitrocellulose filter and were then probed with a *cryIII*-type DNA hybridization probe. The filter was hybridized at 65°C in a buffered aqueous solution containing a radioactively labeled 2.4 kb *cryIIIB* DNA probe. After hybridization, the filter was exposed to X-ray film to

make an autoradiogram. Figure 3 is a photograph of the autoradiogram where the numbers to the left indicate the size, in kb, of the DNA fragments of *B.t.* strain EG5144 that hybridized to the *cryIIIB* probe. These sizes were determined by comparison with the lane labeled "stnd" which contained phage lambda DNA digested with *Hin*dIII and radioactively labelled as size markers. Lanes in Figure 3 marked EG2158, EG5144, EG2838 and EG4961 contain size fractionated DNA fragments from these respective *B.t.* strains, obtained by digestion with the restriction enzyme designated above the individual lanes.

In the lanes for each *B.t* strain in Figure 3, the dark bands represent DNA restriction fragments that hybridized with the *cryIIIB* probe. Visual inspection of Figure 3 shows that the sizes of the *cryIIIB*-hybridizing restriction fragments of *B.t.* strain EG5144 are distinctly different from the sizes of the *cryIIIB*-hybridizing fragments of *B.t.* strains EG2158, EG2838 and EG4961.

In particular, the size of the *cryIIIB*-hybridizing *Ssp*I restriction fragment for *B.t.* strain EG5144 is 3.4 kb, and this is unlike the corresponding *Ssp*I restriction fragments for the other three *B.t.* strains: 2.8 kb for *B.t.* strain EG2158; 2.4 kb for *B.t.* strain EG2838; and 4.5 and 6.0 kb for *B.t.* strain EG4961. Similar differences are apparent for the DNA restriction fragments obtained using *Hin*dIII and *Eco*RI.

)OCID: <WO 9213954A1>

These restriction pattern results suggest that *B.t.* strain EG5144 contains a *cryIII*-type gene that is different from the *cryIIIA*, *cryIIIB* and *cryIIIC(a)* genes of *B.t.* strains EG2158, EG2838 and EG4961, respectively. The *cryIII*-type gene of *B.t.* strain EG5144 has been designated *cryIIIC(b)* (SEQ ID NO:1) by the inventors.

Total DNA from *B.t.* strain EG5144 and *B.t.* strain EG5145 was extracted and digested with six separate restriction enzymes (*Hin*dIII, *Eco*RI, *Acc*I, *Dra*I, *Ssp*I, *Xba*I), and size fractionated by electrophoresis on an agarose gel. The size fractionated DNA restriction fragments were then transferred by blotting to a nitrocellulose filter and were then probed with a *cryIII*-type DNA hybridization probe, specifically a probe containing *cryIIIA*. After hybridization, the filter was exposed to X-ray film to make an autoradiogram. The restriction pattern results were identical for the two *B.t.* strains evaluated, EG5144 and EG5145, which suggests that the two strains contain the same *cryIII*-type gene.

Example 5

Characterization of Crystal Proteins of *B.t.* Strain EG5144

B.t. strain EG5144 was grown in DSMG sporulation medium at room temperature (about 21-25°C) until sporulation and cell lysis had occurred (4 to 5 days growth). The DSMG medium is 0.4% (w/v) Difco nutrient broth, 25 mM K_2HPO_4, 25 mM KH_2PO_4, 0.5 mM $Ca(NO_3)_2$, 0.5 mM

NSDOCID: <WO 9213954A1>

$MgSO_4$, 10 μM $FeSO_4$, 10 μM $MnCl_2$ and 0.5% (w/v) glucose. The sporulated culture of *B.t.* strain EG5144 was observed microscopically to contain free floating, irregularly shaped crystals in addition to *B.t.* spores. Experience has shown that *B.t.* crystals are usually composed of proteins that may be toxic to specific insects. The appearance of the crystals of *B.t.* strain EG5144 differed from the flat, rectangular (or rhomboidal) crystals of *B.t.* strain EG2158, but partially resembled some of the irregularly shaped crystals of *B.t.* strains EG2838 and EG4961.

Spores, crystals and residual lysed cell debris from the sporulated culture of *B.t.* strain EG5144 were harvested by centrifugation. The recovered solids were washed once with aqueous 1N NaCl and twice with TETX (containing 10 mM Tris HCl pH 7.5, 1mM EDTA and 0.005% (w/v) Triton® X-100) and suspended in TETX at a concentration of 50 mg/ml. The washed crystals were specifically solubilized from 250 μg centrifuged fermentation culture solids (containing crystals, spores and some cell debris) by heating the solids mixture in a solubilization buffer (0.14 M Tris pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.1% (v/v) bromophenol blue) at 100°C for 5 minutes. The solubilized crystal proteins were size fractionated by SDS-PAGE. After size fractionation, the proteins were visualized by staining with Coomassie dy . Cultures of

B.t. strains EG4961, EG2158 and EG2838 were processed in an identical manner for purposes of comparison.

Figure 4 shows the results of this protein size fractionation analysis where the numbers to the left indicate the size, in kDa, of the crystal proteins synthesized by *B.t.* strain EG5144. As shown in lane 1, a major protein of approximately 70 kDa and a minor protein of approximately 30 kDa were solubilized from centrifuged fermentation solids containing *B.t.* strain EG5144 spores and crystals. The approximately 70 kDa protein of *B.t.* strain EG5144 appears similar in size to the approximately 70 kDa coleopteran-toxic crystal proteins of *B.t.* strains EG4961 (lane 2), EG2158 (lane 3) and to the approximately 74 kDa coleopteran-toxic crystal protein of *B.t.* strain EG2838 (lane 4).

Previous work by the inventors has shown that the coleopteran-toxic crystal proteins of *B.t.* strains EG4961, EG2158 and EG2838 are each different. The CryIIIC(a) protein of *B.t.* strain EG4961 is coded by the *cryIIIC(a)* gene and has a deduced size of 74,393 Da. The CryIIIA protein of *B.t.* strain EG2158 is coded by the *cryIIIA* gene and has a deduced size of 73,116 Da. The CryIIIB protein of *B.t.* strain EG2838 is coded by the *cryIIIB* gene and has a deduced size of 74,237 Da. As described in Example 6, the coleopteran-toxic crystal protein of *B.t.* strain EG5144 produced by the novel *cryIIIC(b)* gene (SEQ ID NO:1)

is clearly different from the CryIIIA, CryIIIB and CryIIIC(a) proteins.

The minor crystal protein of approximately 30 kDa that is produced by *B.t.* strain EG5144 is roughly similar in size to small crystal proteins produced by B.t. strains EG4961, EG2158 and EG2838. The approximately 30 kDa minor proteins of B.t. strains EG2158, EG2838 and EG4961 appear to be related to each other and none has been found to exhibit measurable insecticidal activity towards coleopteran insects. There is no reason to believe that the approximately 30 kDa protein of *B.t.* strain EG5144 possesses insecticidal activity against coleopteran insects.

Following the procedure of Example 4, further DNA blot analysis revealed that the 2.4 kb *cryIIIB* DNA probe specifically hybridized to a single 7.0 kb *Eco*RI-*Xba*I restriction fragment of *B.t.* strain EG5144 DNA. This result suggested that the 7.0 kb fragment contained the complete *cryIIIC(b)* gene.

The 7.0 kb *Eco*RI-*Xba*I fragment of *B.t.* strain EG5144 was isolated and studies were conducted on the 7.0 kb *Eco*RI-*Xba*I restriction fragment to confirm that the fragment contained a *cryIII*-type gene, in particular, the *cryIIIC(b)* gene. The procedures set forth in Example 6 describe the determination of the nucleotide base sequence of the *cryIIIC(b)* gene (SEQ ID NO:1).

Example 6

Cloning and Sequencing of the *cryIIIC(b)* Gene of *B.t.* Strain EG5144

In order to isolate the 7.0 kb *Eco*RI-*Xba*I fragment described in the previous Example, a plasmid library of *B.t.* strain EG5144 was constructed by ligating size-selected DNA *Eco*RI-*Xba*I restriction fragments from *B.t.* strain EG5144 into the well-known *E.coli* vector pUC18. This procedure involved first obtaining total DNA from *B.t.* strain EG5144 by cell lysis followed by DNA spooling, then double digesting the total DNA with both *Eco*RI and *Xba*I restriction enzymes, electrophoresing the digested DNA through an agarose gel, excising a gel slice containing 4-10 kb size selected fragments of DNA, and electroeluting the size selected *Eco*RI-*Xba*I restriction fragments from the agarose gel slice. These fragments were mixed with the *E.coli* plasmid vector pUC18, which had also been digested with *Eco*RI and *Xba*I. The pUC18 vector carries the gene for ampicillin resistance (Amp^r) and the vector replicates in *E.coli*. T4 DNA ligase and ATP were added to the mixture of size-selected restriction fragments of DNA from *B.t* strain EG5144 and of digested pUC18 vector to allow the pUC18 vector to ligate with the *B.t.* strain EG5144 restriction fragments.

The plasmid library was then transformed into *E. coli* cells, a host organism lacking the gene of interest, as follows. After ligation, the DNA mixture was incubated with an ampicillin sensitive *E. coli* host strain, *E. coli*

strain DH5α, that had been treated with $CaCl_2$ to allow the cells to take up the DNA. *E. coli*, specifically strain DH5α, was used as the host strain because these cells are easily transformed with recombinant plasmids and because *E. coli* strain DH5α does not naturally contain genes for *B.t.* crystal proteins. Since pUC18 confers resistance to ampicillin, all host cells acquiring a recombinant plasmid would become ampicillin resistant. After exposure to the recombinant plasmids, the *E. coli* host cells were spread on agar medium that contained ampicillin. After incubation overnight at a temperature of 37°C, several thousand *E. coli* colonies grew on the ampicillin-containing agar from those cells which harbored a recombinant plasmid. These *E. coli* colonies were then blotted onto nitrocellulose filters for subsequent probing.

The radioactively labeled 2.4 kb *cryIIIB* gene was then used as a DNA probe under conditions that permitted the probe to bind specifically to those transformed host colonies that contained the 7.0 kb *Eco*RI-*Xba*I fragment of DNA from *B.t.* strain EG5144. Several *E. coli* colonies specifically hybridized to the 2.4 kb *cryIIIB* probe. One *cryIIIB*-hybridizing colony, designated *E. coli* strain EG7236, was studied further. *E. coli* strain EG7236 contained a recombinant plasmid, designated pEG271, which consisted of pUC18 plus the inserted *Eco*RI-*Xba*I restriction fragment of DNA from *B.t.* strain EG5144 of

approximately 7.0 kb. The *cryIIIB* probe specifically hybridized to the 7.0 kb DNA fragment insert in pEG271. A restriction map of pEG271 is shown in Figure 5.

The 7.0 kb fragment of pEG271 contained *Hin*dIII fragments of 2.4 kb and 3.8 kb, and a *Bam*HI-*Xba*I fragment of 4.0 kb that specifically hybridized with the *cryIIIB* probe. The 2.4 kb *Hin*dIII fragment was subcloned into the DNA sequencing vector M13mp18. The 4.0 kb *Bam*HI-*Xba*I fragment was subcloned into the DNA sequencing vectors M13mp18 and M13mp19.

The nucleotide base sequence of a substantial part of each subcloned DNA fragment was determined using the standard Sanger dideoxy method. For each subcloned fragment, both DNA strands were sequenced by using sequence-specific 17-mer oligonucleotide primers to initiate the DNA sequencing reactions. Sequencing revealed that the 7.0 kb fragment contained an open reading frame and, in particular, a new *cryIII*-type gene. This new gene, designated *cryIIIC(b)* (SEQ ID NO:1), is significantly different from the *cryIIIA* gene. As indicated below, the *cryIIIC(b)* gene is also clearly distinct from the *cryIIIB* gene.

The DNA sequence of the *cryIIIC(b)* gene (SEQ ID NO:1) and the deduced amino acid sequence of the CryIIIC(b) protein (SEQ ID NO:2) encoded by the *cryIIIC(b)* gene are shown in Figure 1. The protein coding portion of the *cryIIIC(b)* gene (SEQ ID NO:1) is defined by the

NSDOCID: <WO 9213954A1>

nucleotides starting at position 144 and ending at position 2099. The probable ribosome binding site is indicated as "RBS" in Figure 1-1. The size of the CryIIIC(b) protein (SEQ ID NO:2) encoded by the *cryIIIC(b)* gene, as deduced from the open reading frame of the *cryIIIC(b)* gene (SEQ ID NO:1), is 74,265 Da (652 amino acids). It should be noted that the apparent size of the CryIIIC(b) protein, as determined from SDS-PAGE, is approximately 70 kDa. Therefore, the CryIIIC(b) protein (SEQ ID NO:2) will be referred to in this specification as being approximately 70 kDa in size.

The size of the prior art CryIIIA protein has previously been deduced to be 73,116 Da (644 amino acids). The size of the CryIIIB protein has previously been determined to be 74,237 Da (651 amino acids).

DNA sequencing revealed the presence of a *Hin*dIII restriction site within the *cryIIIC(b)* gene and a *Ssp*I restriction site downstream of the *cryIIIC(b)* gene (See Figures 1-2 and 1-3 respectively). Knowledge of the locations of these restriction sites permitted the precise determination of the location and orientation of the *cryIIIC(b)* gene within the 7.0 kb fragment as indicated by the arrow in Figure 5.

The computer program of Korn and Queen (L.J. Korn and C. Queen, "Analysis of Biological Sequences on Small Computers," *DNA*, 3, pp. 421-436 (1984)) was used to compare the sequences of the *cryIIIC(b)* gene (SEQ ID NO:1)

)OCID: <WO 9213954A1>

to the *cryIIIB* and *cryIIIA* genes and to compare the deduced amino acid sequences of their respective CryIIIC(b), CryIIIB and CryIIIA proteins.

The nucleotide base sequence of the *cryIIIC(b)* gene (SEQ ID NO:1) was 96% positionally identical with the nucleotide base sequence of the *cryIIIB* gene and only 76% positionally identical with the nucleotide base sequence of the *cryIIIA* gene. Thus, although the *cryIIIC(b)* gene (SEQ ID NO:1) is related to the *cryIIIB* and *cryIIIA* genes, it is clear that the *cryIIIC(b)* gene is distinct from the *cryIIIB* gene and substantially different from the *cryIIIA* gene.

The deduced amino acid sequence of the CryIIIC(b) protein (SEQ ID NO:2) was found to be 95% positionally identical to the deduced amino acid sequence of the CryIIIB protein, but only 68% positionally identical to the deduced amino acid sequence of the CryIIIA protein. These differences, together with the differences in insecticidal activity as set forth below, clearly show that the CryIIIC(b) protein encoded by the *cryIIIC(b)* gene (SEQ ID NO:1) is a different protein from the CryIIIB protein or the CryIIIA protein.

Moreover, while not wishing to be bound by any theory, based on a comparison of the amino acid sequences of the CryIIIC(b) protein (SEQ ID NO:2) with other CryIII-type proteins known to the inventors, it is believed that the following amino acid residues may be of significance

NSDOCID: <WO 9213954A1>

for the enhanced corn rootworm toxicity of the CryIIIC(b) protein, where the numbers following the accepted abbreviations for the amino acids indicate the position of the amino acid in the sequence illustrated in Figure 1 and identified in SEQ ID NO:2: His9, His231, Gln339, Ser352, Asn446, His449, Val450, Gly451, Ile600 and Thr624. These amino acid residues were selected as being of probable significance for the corn rootworm toxicity of the CryIIIC(b) protein (SEQ ID NO:2) because, after studying the amino acid sequences of several other CryIII proteins, the amino acids at the indicated positions fairly consistently showed different amino acids than those indicated for the CryIIIC(b) protein.

Based on the same studies, it is also believed that site directed mutagenesis of the *cryIIIC(b)* gene (SEQ ID NO:1) may result in improved or enhanced corn rootworm toxicity for the resultant protein where one or more of the following amino acid modifications are effected: Pro21 to Gly; Asp97 to Asn; Val289 to Ile; Ser352 to Phe; 417Ile to Val; Phe419 to Leu; Gly451 to Ser; Ile590 to Leu; Ile600 to Lys; Thr624 to Lys.

As is well understood in the art, other changes in the *cryIIIC(b)* gene (SEQ ID NO:1) may be made, via site directed mutagenesis or gene truncation or the like, that could yield a toxic protein which possesses essentially similar insecticidal activity (to corn rootworm and other coleopteran insects) as that exhibited by the CryIIIC(b)

protein (SEQ ID NO:2). Modifications to the *cryIIIC(b)* gene (SEQ ID NO:1) and CryIIIC(b) protein (SEQ ID NO:2) such as described above are intended to be within the scope of the claimed invention.

Example 7

Expression of the Cloned *cryIIIC(b)* Gene

Studies were conducted to determine the production of the CryIIIC(b) protein (SEQ ID NO:2) by the *cryIIIC(b)* gene (SEQ ID NO:1).

Table 2 summarizes the relevant characteristics of the *B.t.* and *E. coli* strains and plasmids used during these procedures. A plus ($^+$) indicates the presence of the designated element, activity or function and a minus ($^-$) indicates the absence of the same. The designations ^{s} and ^{r} indicate sensitivity and resistance, respectively, to the antibiotic with which each is used. The abbreviations used in the Table have the following meanings: Amp (ampicillin); Cm (chloramphenicol); Cry (crystalliferous); Tc (tetracycline).

Table 2

Strains and Plasmids

Strains	Relevant characteristics
B. thuringiensis	
HD73-26	Cry^-, Cm^s
EG7237	HD73-26 harboring pEG272 (*cryIIIC(b)*$^+$)

ISDOCID: <WO 9213954A1>

EG5144	*cryIIIC(b)*$^+$
E. coli	
DH5α	Cry$^-$, Amp^S
GM2163	Cry$^-$, Amp^S
EG7236	DH5α harboring pEG271 (*cryIIIC(b)*$^+$)

Plasmids

pUC18	Amp^r, Cry$^-$, *E. coli* vector
pNN101	Cm^r, Tc^r, Cry$^-$, *Bacillus* vector
pEG271	Amp^r, *cryIIIC(b)*$^+$ *E. coli* recombinant plasmid consisting of the 7.0 kb *Eco*RI-*Xba*I *cryIIIC(b)*$^+$ fragment of *B.t.* strain EG5144 ligated into the *Eco*RI-*Xba*I sites of pUC18
pEG272	Tc^r, Cm^r, *cryIIIC(b)*$^+$ *Bacillus-E. coli* recombinant plasmid consisting of the *Bacillus* vector pNN101 ligated into the *Sph*I site of pEG271.

E. coli cells harboring plasmid pEG271 described in Example 6 were analyzed and found not to produce detectable amounts of the 70 kDa CryIIIC(b) crystal protein.

Experience has shown that cloned *B.t.* crystal genes are poorly expressed in *E. coli* and highly expressed in *B.t.* from their respective native promoter sequences.

DOCID: <WO 9213954A1>

Recombinant plasmid pEG271, constructed as set forth in Example 6 and shown in Figure 5, will replicate in *E. coli*, but will not replicate in *B.t.* To achieve a high level of expression of the cloned *cryIIIC(b)* gene, th *Bacillus* vector pNN101 (Tc^r Cm^r Cry^-) that is capable of replicating in *B.t.* was ligated into the *Sph*I site of pEG271. The resultant plasmid was designated pEG272. Details of the construction of plasmid pEG272 and its subsequent use to transform *B.t.* are described below.

The isolated plasmid pEG271 DNA was digested with *Sph*I and was then mixed with the *Bacillus* vector pNN101 that had also been digested with *Sph*I. T4 DNA ligase and ATP were added to the mixture to allow pEG271 to ligate into the *Sph*I site of the pNN101 vector.

After ligation, the DNA mixture was added to a suspension of *E. coli* strain DH5α cells that had been treated with calcium chloride to permit the cells to take up plasmid DNA. After exposure to the recombinant plasmids, the *E. coli* host cells were spread on an agar medium containing tetracycline. Only cells that had taken up a plasmid consisting of pEG271 ligated into the *Sph*I site of pNN101 would grow on the tetracycline agar medium whereas cells that had not absorbed the plasmid would not grow.

Plasmid was isolated from one tetracycline resistant colony, digested with *Sph*I, and electrophoresed through an agarose gel. The plasmid consisted of two *Sph*I DNA

fragments of 5.8 kb and 9 kb corresponding to plasmids pNN101 and pEG271, respectively. This plasmid was designated pEG272. A restriction map of pEG272 is shown in Figure 6. Plasmid pEG272 was then used to transform cells of *E. coli* strain GM2163 made competent by the calcium chloride procedure described earlier in Example 6. *E. coli* strain GM2163 is a crystal negative (Cry^-) and ampicillin sensitive (Amp^S) strain, constructed by the procedures of M.G. Marinus et al. in *Mol.Gen.Genet., 192,* pp.288-289 (1983).

Plasmid pEG272 was then isolated from the transformed *E. coli* strain GM2163, using the procedures described above. The isolated plasmid pEG272 was next transformed by electroporation into *B.t.* strain HD73-26. Cells of *B.t.* strain HD73-26 are crystal-negative (Cry^-) and chloramphenicol sensitive (Cm^S). Using a BioRad Gene Pulser™ apparatus to carry out the electroporation, cells of *B.t.* strain HD73-26 in suspension were induced to take up pEG272 which was also added to the mixture.

After electroporation, the transformed *B.t.* cells were spread onto an agar medium containing 5 μg chloramphenicol and were incubated about 16-18 hours at 30°C. Cells that had taken up plasmid pEG272 would grow into colonies on the chloramphenicol agar medium whereas cells that had not absorbed the plasmid would not grow. One Cm^r colony, designated *B.t.* strain EG7237, contained a

DOCID: <WO 9213954A1>

plasmid whose restriction pattern appeared identical to that of pEG272.

Cells of B.t. strain EG7237 were grown in a sporulation medium containing chloramphenicol (3 μg/ml) at 22-25°C until sporulation and cell lysis had occurred (4-5 days). Microscopic examination revealed that the sporulated culture of *B.t.* strain EG7237 contained spores and small free floating irregularly shaped crystals. These crystals resembled the small, irregularly-shaped crystals observed with a sporulated culture of *B.t.* strain EG5144 that had been prepared in a similar manner.

Spores, crystals and cell debris from the sporulated fermentation culture of *B.t.* strain EG7237 were harvested by centrifugation. The centrifuge pellet was washed onc with 1N aqueous NaCl and twice with TETX (10 mM Tris·HCl pH 7.5, 1 mM EDTA, 0.005% (w/v) Triton® X-100), and the pellet suspended in TETX at a concentration of 50 mg pellet/ml TETX.

The crystals in the centrifuge pellet suspension were solubilized by heating a portion of the centrifuge suspension (containing 250 μg pellet solids) in solubilization buffer (0.14 M Tris pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.1% (w/v) bromophenol blue) at 100°C for 5 minutes. After crystal solubilization had occurred, the mixture was applied to an SDS-polyacryamide gel and the solubilized proteins in the mixture were size fractionated by

ISDOCID: <WO 9213954A1>

electrophoresis. After size fractionization, the proteins were visualized by staining with Coomassie dye. A photograph of the Coomassie stained gel is shown in Figure 7.

Lane 3 of the gel in Figure 7 shows that *B.t.* strain EG7237 produced a major protein of approximately 70 kDa and a minor protein of approximately 30 kDa. These proteins appeared to be identical in size with the major approximately 70 kDa protein and the minor approximately 30 kDa protein produced by *B.t.* strain EG5144, which ar shown in the lane 1 of Figure 7 and which were prepared in a manner identical to *B.t.* strain EG7237. This result indicates that the 7.0 kb fragment of pEG272 contains two crystal protein genes: one for the approximately 70 kDa protein and one for the approximately 30 kDa protein.

The gene encoding the approximately 70 kDa protein is the *cryIIIC(b)* gene, and its encoded protein is the insecticidal CryIIIC(b) protein. The DNA sequence for the *cryIIIC(b)* gene (SEQ ID NO:1) and the amino acid sequence for its corresponding deduced protein (SEQ ID NO:2) are shown in Figure 1.

B.t. strain EG7237 produced approximately three times more 70 kDa protein, on a weight basis, than did *B.t.* strain EG5144, as is evident from the protein bands in Figure 7. Production of the minor 30 kDa protein in recombinant *B.t.* strain EG7237 was also increased, as compared with *B.t.* strain EG5144.

The following Examples 8-11 describe the manner in which the insecticidal activities of *B.t.* strain EG5144, *B.t.* strain EG7237, and the CryIIIC(b) protein made by these strains were determined.

Example 8

Insecticidal Activity of *B.t.* Strain EG7237 and its CryIIIC(b) Protein Against Southern Corn Rootworm and Colorado Potato Beetl

The insecticidal activity of recombinant *B.t.* strain EG7237, which contains the *cryIIIC(b)* gene (SEQ ID NO:1) that produces the CryIIIC(b) toxin protein (SEQ ID NO:2), was determined against southern corn rootworm (*Diabrotica undecimpunctata howardi*) and Colorado potato beetle (*Leptinotarsa decemlineata*).

For comparison, two other recombinant *B.t.* strains containing *cryIII*-type toxin genes in a *B.t.* strain HD73-26 background were also included in the bioassay study. These were recombinant *B.t.* strain EG7235, which contains the *cryIIIA* gene that produces the CryIIIA toxin protein, and recombinant *B.t.* strain EG7225, which contains the *cryIIIB* gene that produces the CryIIIB toxin protein.

The three *B.t.* strains were grown in liquid sporulation media at 30°C until sporulation and cell lysis had occurred. The fermentation broth was concentrated by microfiltration. The concentrated fermentation broth was th n freeze dried to prepare a *B.t.* powder suitable for

SDOCID: <WO 9213954A1>

insect bioassay. The amount of CryIII-type toxin protein in each of the *B.t.* powders was quantified using standard SDS-PAGE techniques.

First instar southern corn rootwom larvae were bioassayed via surface contamination of an artificial diet similar to Marrone et al., *J.Econ.Entomol.*, 78, pp.290-293 (1985), but without formalin. Each bioassay consisted of eight serial aqueous dilutions with aliquots applied to the surface of the diet in a bioassay tray. Each 2 ml well of the bioassay tray contained 1 ml diet having a surface area of 175 mm^2. After the diluent (an aqueous 0.005% Triton® X-100 solution) had evaporated, the insect larvae were placed on the diet and incubated at 28°C. Thirty-two larvae were tested per dose. Mortality was scored after 7 days. A control, consisting of diluent only, was also included in the bioassay study.

First instar Colorado potato beetle larvae were tested using similar techniques, except for the substitution in the artificial diet of BioServe's No. 9830 insect diet with potato flakes added. Thirty-two larvae were tested per dose, and mortality was scored at three days instead of seven days.

The results of the bioassay study are shown below in Table 3, where insecticidal activity is reported as PLC_{50} values, the concentration of CryIII-type protein required to kill 50% of the insects tested. Four replications per dose were us d in the bioassay studies for both insects

tested. Data from each of the replicated bioassays were pooled for probit analysis (R.J. Daum, *Bull.Entomol.Soc.Am., 16*, pp.10-15 (1970)) with mortality corrected for control death, the control being the diluent only (W.S. Abbott, *J.Econ.Entomol., 18*, pp.265-267 (1925)). Results are shown as the dose amount of CryIII-type protein (in ng CryIII protein per mm^2 of diet surface) resulting in PLC_{50}. Confidence intervals, at 95%, are given within parentheses below the PLC_{50} values.

Table 3

Insecticidal Activity of Recombinant *B.t.* Strains EG7237, EG7235 and EG7225

B.t. Strain	CryIII Protein	CryIII Protein Concentration (%)	Southern Corn Rootworm PLC_{50} (ng CryIII protein/mm^2)	Colorado Potato Beetle PLC_{50} (ng CryIII protein/mm^2)
B.t. EG7237	CryIIIC(b)	7.2	1548 (1243-1992)	6.92 (5.15 - 9.10)
B.t. EG7235	CryIIIA	28.4	6% control at 4570	0.34 (0.30 - 0.39)
B.t. EG7225	CryIIIB	9.4	20% control at 4570	1.26 (1.07 - 1.46)

The results of this bioassay study demonstrate that *B.t.* strain EG7237 which produces the CryIIIC(b) toxin protein (SEQ ID NO:2) is insecticidal to southern corn rootworm. In contrast, the CryIIIA and CryIIIB toxin proteins of *B.t.* strains EG7235 and EG7225, respectively, appear to have no measurable activity against this insect at the highest dose level tested.

All three of the *B.t.* strains exhibit insecticidal activity against Colorado potato beetle larvae, with the CryIIIA toxin protein of *B.t.* strain EG7235 being significantly more potent than the CryIIIC(b) toxin protein (SEQ ID NO:2) of *B.t.* strain EG7237 and with the CryIIIB toxin protein of *B.t.* strain EG7225 having insecticidal activity falling between that shown for CryIIIA and CryIIIC(b).

These results suggest that the insecticidal activity of specific CryIII-type toxin proteins varies for different insect genera within the order Coleoptera.

Example 9

Insecticidal Activity of *B.t.* Strain EG7237 and its CryIIIC(b) Protein Against Mexican Bean Beetle

The insecticidal activity of recombinant *B.t.* strain EG7237, evaluated in Example 8, was also determined against Mexican bean beetle (*Epilachna varivestis*). As in Example 8, recombinant *B.t.* strains EG7235 and EG7225 were

USDOCID: <WO 9213954A1>

included for comparison, and all *B.t.* powders were prepared as in Example 8.

First instar Mexican bean beetle larvae were bioassayed by a leaf dip procedure, since a suitable artificial diet is not available for this insect. Soybean leaves were dipped into known treatment concentrations of the *B.t.* powder suspended in an aqueous 0.1% Triton® X-100 solution. After excess material had dripped off, the leaves were allowed to dry. Leaves dipped in 0.1% Triton® X-100 served as untreated controls. Twenty insect larvae were confined to a petri dish with treated leaves, incubated at 25°C, and allowed to feed for three days, at which time mortality was scored.

The results of the bioassay study are shown below in Table 4, where insecticidal activity is reported as PLC_{50} values, the concentration of CryIII-type protein required to kill 50% of the insects tested. The data were handled as described in Example 8, for Table 3. Results are shown as the dose amount of CryIII-type protein (in mg CryIII protein/ml solution used in the leaf dip) resulting in PLC_{50}. Confidence intervals, at 95%, are given within parentheses following the PLC_{50} values.

Table 4

Insecticidal Activity of *B.t.* Strains EG7237, EG7235 and EG7225 Against Mexican Bean Beetle

B.t. Strain	CryIII Protein	No. of Replications	PLC_{50} (mg CryIIIprotein/ml)
B.t. EG7237	CryIIIC(b)	4	4.2 (2.5-6.5)
B.t. EG7235	CryIIIA	4	16% control at 60
B.t. EG7225	CryIIIB	4	51.8 (31-209)

The results of this bioassay study demonstrate that *B.t.* strain EG7237 which produces the CryIIIC(b) toxin protein (SEQ ID NO:2) is significantly more insecticidal to Mexican bean beetle than the CryIIIB-producing *B.t.* strain EG7225. *B.t.* strain EG7235 which produces CryIIIA toxin protein exhibited no measurable insecticidal activity at the highest dose tested.

These results are further evidence that the insecticidal activity of specific CryIII-type toxin proteins varies widely for insect genera within the order Coleoptera.

Example 10

Insecticidal Activity of *B.t.* Strain EG5144 Against Southern Corn Rootworm

The insecticidal activity of *B.t.* strain EG5144 was evaluated against Southern corn rootworm (*Diabrotica und cimpunctata howardi*). For comparison, *B.t.* strain

EG4961 which produces the CryIIIC(a) toxin protein was included in the bioassay study.

The bioassay procedure for southern corn rootworm in this Example determined PLC_{50} values, the concentration of CryIII-type protein required to kill 50% of the insects tested. The procedure was similar to the artificial diet bioassay carried out in the previous Example, using thirty-two first instar southern corn rootworm larvae per dose. Data from each of the replicated bioassays were pooled for probit analysis (R.J. Daum, *Bull.Entomol.Soc.Am.*, *16*, pp.10-15 (1970)) with mortality corrected for control death, the control being the diluent only (W.S. Abbott, *J.Econ.Entomol.*, *18*, pp.265-267 (1925)). Results are reported for two separate tests as the dose amount of CryIII-type protein (ng CryIII protein per mm^2 of diet surface) resulting in PLC_{50}. Confidence intervals, at 95%, are given within parentheses following the PLC_{50} values. In Test 1 four replications per dose were used, and in Test 2, carried out at a later date, two replications were used.

The *B.t.* strains used in this Example were prepared as described for the *B.t.* strains in Example 8, except that the fermentation broth was concentrated by centrifugation.

The results of this bioassay study with southern corn rootworm are shown below in Table 5.

Table 5

Insecticidal Activity of *B.t.* Strains EG5144 and EG4961 Against Southern Corn Rootworm

B.t. Strain	CryIII Protein		CryIII Protein Concentration (%)	PLC_{50} (ng CryIII protein/mm^2)
B.t. EG5144	CryIIIC(b)	Test 1:	4.0	944 (690-1412)
		Test 2:	6.4	1145 (773-2185)
B.t. EG4961	CryIIIC(a)	Test 1:	11.6	102 (86-119)
		Test 2:	11.6	165 (121-220)

This bioassay study demonstrates that both *B.t.* strain EG5144 and *B.t.* strain EG4961, which produce CryIIIC-type proteins, provide quantifiable insecticidal activity against southern corn rootworm.

Example 11

Insecticidal Activity of *B.t.* Strain EG5144 Against Japanese Beetle Larvae

The insecticidal activity of *B.t.* strain EG5144 was evaluated against Japanese beetle larvae, also known as white grubs (*Popillia japonica*). For comparison, *B.t.* strain EG4961 which produces the CryIIIC(a) toxin protein was included in the bioassay study, as were *B.t.* strain EG2158 which produces the CryIIIA toxin protein and *B.t.* strain EG2838 which produces the CryIIIB toxin protein.

The bioassay procedure in this Example was a screening assay, at a single dose of CryIII-type protein

ISDOCID: <WO 9213954A1>

in a diet incorporation assay (1 mg CryIII-type protein per ml diet). *B.t.* powder to be tested, suspended in a diluent (an aqueous 0.005% Triton® X-100 solution) was incorporated into 100 ml of hot (50°-60°C), liquid artificial diet (based on the insect diet described by Ladd, Jr. in *J.Econ.Entomol.*, *79*, pp.668-671 (1986)). The mixture was allowed to solidify in petri dishes, and one 19 mm diameter plug of this material then placed in each well of a plastic ice cube tray. One grub was introduced per well of the trays, the wells were covered with moist germination paper overlaid with aluminum foil, and the trays were held at 25°C for seven days before mortality was scored. The insects tested were third instar Japanese beetle grubs. Two replications of sixteen insects each were carried out in this study.

The results of this screening bioassay study are shown below in Table 6, where insecticidal activity is reported as percentage insect mortality, with the mortality being corrected for control death, the control being diluent only incorporated into the diet plug. Results were obtained at a single dose rate of CryIII-type protein: 1 mg CryIII-type protein per ml of diet; percentage CryIII-type protein present in the respective *B.t.* powders is also shown in Table 6.

)OCID: <WO 9213954A1>

NSDOCID: <WO 9213954A1>

Table 6

Insecticidal Activity of B.t. Strains EG5144, EG4961, EG2158 and EG2838 Against Japanese Beetle Grubs

B.t. Strain	CryIII Protein	CryIII-type Protein in B.t. Powder (wt. %)	CryIII-type Protein Dose (mg CryIII-type protein/ml diet)	Insect Mortality (%)
B.t. EG5144	CryIIIC(b)	5.4	1	62.5
B.t. EG4961	CryIIIC(a)	18.0	1	9
B.t. EG2158	CryIIIA	14.0	1	44
B.t. EG2838	CryIIIB	7.2	1	48

The insecticidal performance against Japanese beetle grubs of *B.t.* strain EG5144 with its CryIIIC(b) toxin protein (SEQ ID NO:2) is clearly superior to that of *B.t.* strain EG4961 with its CryIIIC(a) protein.

With respect to *B.t.* strains EG2158 and *B.t.* strain EG2838, *B.t.* strain EG5144 exhibited superior insecticidal performance against Japanese beetle grubs.

B.t. strain EG5145, whose characteristics are similar to those of *B.t.* strain EG5144, has been found to exhibit insecticidal activity against Japanese beetle grubs equivalent to that of *B.t.* strain EG5144, although the bioassay data are not presented in this Example 11.

Microorganism Deposits

To assure the availability of materials to those interested members of the public upon issuance of a patent on the present application, deposits of the following microorganisms were made prior to the filing of present application with the ARS Patent Collection, Agricultural Research Culture Collection, Northern Regional Research Laboratory (NRRL), 1815 North University Street, Peoria, Illinois 61604, as indicated in the following Table 7:

Table 7

Bacterial Strain	NRRL Accession No.	Date of Deposit
B.t. EG2158	B-18213	April 29, 1987
B.t. HD73-26	B-18508	June 12, 1989
B.t. EG2838	B-18603	February 8, 1990
B.t. EG5144	B-18655	May 22, 1990
B.t. EG7237	B-18736	October 17, 1990
E.coli EG7236	B-18662	June 6, 1990
B.t. EG5145	B-18920	November 21, 1991

These microorganism deposits were made under the provisions of the "Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure". All restrictions on the availability to the public of these deposited microorganisms will be irrevocably removed upon issuance of a patent based on this application.

The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification as indicating the scope of the invention.

NSDOCID: <WO 9213954A1>

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Donovan, William P.
Rupar, Mark J.
Slaney, Annette C.

(ii) TITLE OF INVENTION: BACILLUS THURINGIENSIS cryIIIC(b) TOXIN GENE AND PROTEIN TOXIC TO COLEOPTERAN INSECTS

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Panitch Schwarze Jacobs & Nadel c/o A.S. Nadel
(B) STREET: 1601 Market Street, 36th Floor
(C) CITY: Philadelphia
(D) STATE: Pennsylvania
(E) COUNTRY: U.S.A.
(F) ZIP: 19103

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/649,562
(B) FILING DATE: 31-JAN-1991

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Egolf, Christopher
(B) REGISTRATION NUMBER: 27633
(C) REFERENCE/DOCKET NUMBER: 7205-29 P1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 215-757-1590

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2430 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS

)OCID: <WO 9213954A1>

(B) LOCATION: 144..2099

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
CCATATACAA CTTATCAGGA AGGGGGGGAT GCACAAAGAA GAAAAGAATA AGAAGTGAAT      60

GTTTATAATG TTCAATAGTT TTATGGGAAG GCATTTTATC AGGTAGAAAG TTATGTATTA     120

TGATAAGAAT GGGAGGAAGA AAA ATG AAT CCA AAC AAT CGA AGT GAA CAT          170
                          Met Asn Pro Asn Asn Arg Ser Glu His
                            1               5

GAT ACG ATA AAG GTT ACA CCT AAC AGT GAA TTG CCA ACT AAC CAT AAT        218
Asp Thr Ile Lys Val Thr Pro Asn Ser Glu Leu Pro Thr Asn His Asn
 10                  15                  20                  25

CAA TAT CCT TTA GCT GAC AAT CCA AAT TCG ACA CTA GAA GAA TTA AAT        266
Gln Tyr Pro Leu Ala Asp Asn Pro Asn Ser Thr Leu Glu Glu Leu Asn
                 30                  35                  40

TAT AAA GAA TTT TTA AGA ATG ACT GAA GAC AGT TCT ACG GAA GTG CTA        314
Tyr Lys Glu Phe Leu Arg Met Thr Glu Asp Ser Ser Thr Glu Val Leu
             45                  50                  55

GAC AAC TCT ACA GTA AAA GAT GCA GTT GGG ACA GGA ATT TCT GTT GTA        362
Asp Asn Ser Thr Val Lys Asp Ala Val Gly Thr Gly Ile Ser Val Val
         60                  65                  70

GGG CAG ATT TTA GGT GTT GTA GGA GTT CCA TTT GCT GGG GCA CTC ACT        410
Gly Gln Ile Leu Gly Val Val Gly Val Pro Phe Ala Gly Ala Leu Thr
     75                  80                  85

TCA TTT TAT CAA TCA TTT CTT GAC ACT ATA TGG CCA AGT GAT GCT GAC        458
Ser Phe Tyr Gln Ser Phe Leu Asp Thr Ile Trp Pro Ser Asp Ala Asp
 90                  95                  100                 105

CCA TGG AAG GCT TTT ATG GCA CAA GTT GAA GTA CTG ATA GAT AAG AAA        506
Pro Trp Lys Ala Phe Met Ala Gln Val Glu Val Leu Ile Asp Lys Lys
                110                 115                 120

ATA GAG GAG TAT GCT AAA AGT AAA GCT CTT GCA GAG TTA CAG GGT CTT        554
Ile Glu Glu Tyr Ala Lys Ser Lys Ala Leu Ala Glu Leu Gln Gly Leu
            125                 130                 135

CAA AAT AAT TTC GAA GAT TAT GTT AAT GCG TTA AAT TCC TGG AAG AAA        602
Gln Asn Asn Phe Glu Asp Tyr Val Asn Ala Leu Asn Ser Trp Lys Lys
        140                 145                 150

ACA CCT TTA AGT TTG CGA AGT AAA AGA AGC CAA GAT CGA ATA AGG GAA        650
Thr Pro Leu Ser Leu Arg Ser Lys Arg Ser Gln Asp Arg Ile Arg Glu
    155                 160                 165

CTT TTT TCT CAA GCA GAA AGT CAT TTT CGT AAT TCC ATG CCG TCA TTT        698
Leu Phe Ser Gln Ala Glu Ser His Phe Arg Asn Ser Met Pro Ser Phe
170                 175                 180                 185

GCA GTT TCC AAA TTC GAA GTG CTG TTT CTA CCA ACA TAT GCA CAA GCT        746
Ala Val Ser Lys Phe Glu Val Leu Phe Leu Pro Thr Tyr Ala Gln Ala
                190                 195                 200
```

```
GCA AAT ACA CAT TTA TTG CTA TTA AAA GAT GCT CAA GTT TTT GGA GAA        794
Ala Asn Thr His Leu Leu Leu Leu Lys Asp Ala Gln Val Phe Gly Glu
            205                 210                 215

GAA TGG GGA TAT TCT TCA GAA GAT GTT GCT GAA TTT TAT CAT AGA CAA        842
Glu Trp Gly Tyr Ser Ser Glu Asp Val Ala Glu Phe Tyr His Arg Gln
        220                 225                 230

TTA AAA CTT ACG CAA CAA TAC ACT GAC CAT TGT GTC AAT TGG TAT AAT        890
Leu Lys Leu Thr Gln Gln Tyr Thr Asp His Cys Val Asn Trp Tyr Asn
    235                 240                 245

GTT GGA TTA AAT GGT TTA AGA GGT TCA ACT TAT GAT GCA TGG GTC AAA        938
Val Gly Leu Asn Gly Leu Arg Gly Ser Thr Tyr Asp Ala Trp Val Lys
250                 255                 260                 265

TTT AAC CGT TTT CGC AGA GAA ATG ACT TTA ACT GTA TTA GAT CTA ATT        986
Phe Asn Arg Phe Arg Arg Glu Met Thr Leu Thr Val Leu Asp Leu Ile
                270                 275                 280

GTA CTT TTC CCA TTT TAT GAT GTT CGG TTA TAC TCA AAA GGT GTT AAA       1034
Val Leu Phe Pro Phe Tyr Asp Val Arg Leu Tyr Ser Lys Gly Val Lys
            285                 290                 295

ACA GAA CTA ACA AGA GAC ATT TTT ACG GAT CCA ATT TTT TCA CTC AAT       1082
Thr Glu Leu Thr Arg Asp Ile Phe Thr Asp Pro Ile Phe Ser Leu Asn
        300                 305                 310

ACT CTT CAG GAG TAT GGA CCA ACT TTT TTG AGT ATA GAA AAC TCT ATT       1130
Thr Leu Gln Glu Tyr Gly Pro Thr Phe Leu Ser Ile Glu Asn Ser Ile
    315                 320                 325

CGA AAA CCT CAT TTA TTT GAT TAT TTA CAG GGT ATT GAA TTT CAT ACG       1178
Arg Lys Pro His Leu Phe Asp Tyr Leu Gln Gly Ile Glu Phe His Thr
330                 335                 340                 345

CGT CTT CAA CCT GGT TAC TCT GGG AAA GAT TCT TTC AAT TAT TGG TCT       1226
Arg Leu Gln Pro Gly Tyr Ser Gly Lys Asp Ser Phe Asn Tyr Trp Ser
                350                 355                 360

GGT AAT TAT GTA GAA ACT AGA CCT AGT ATA GGA TCT AGT AAG ACA ATT       1274
Gly Asn Tyr Val Glu Thr Arg Pro Ser Ile Gly Ser Ser Lys Thr Ile
            365                 370                 375

ACT TCC CCA TTT TAT GGA GAT AAA TCT ACT GAA CCT GTA CAA AAG TTA       1322
Thr Ser Pro Phe Tyr Gly Asp Lys Ser Thr Glu Pro Val Gln Lys Leu
        380                 385                 390

AGC TTT GAT GGA CAA AAA GTT TAT CGA ACT ATA GCT AAT ACA GAC GTA       1370
Ser Phe Asp Gly Gln Lys Val Tyr Arg Thr Ile Ala Asn Thr Asp Val
    395                 400                 405

GCG GCT TGG CCG AAT GGC AAG ATA TAT TTT GGT GTT ACG AAA GTT GAT       1418
Ala Ala Trp Pro Asn Gly Lys Ile Tyr Phe Gly Val Thr Lys Val Asp
410                 415                 420                 425

TTT AGT CAA TAT GAT GAT CAA AAA AAT GAA ACT AGT ACA CAA ACA TAT       1466
Phe Ser Gln Tyr Asp Asp Gln Lys Asn Glu Thr Ser Thr Gln Thr Tyr
                430                 435                 440
```

DOCID: <WO 9213954A1>

```
GAT TCA AAA AGA AAC AAT GGC CAT GTA GGT GCA CAG GAT TCT ATT GAC      1514
Asp Ser Lys Arg Asn Asn Gly His Val Gly Ala Gln Asp Ser Ile Asp
            445                 450                 455

CAA TTA CCA CCA GAA ACA ACA GAT GAA CCA CTT GAA AAA GCA TAT AGT      1562
Gln Leu Pro Pro Glu Thr Thr Asp Glu Pro Leu Glu Lys Ala Tyr Ser
        460                 465                 470

CAT CAG CTT AAT TAC GCG GAA TGT TTC TTA ATG CAG GAC CGT CGT GGA      1610
His Gln Leu Asn Tyr Ala Glu Cys Phe Leu Met Gln Asp Arg Arg Gly
    475                 480                 485

ACA ATT CCA TTT TTT ACT TGG ACA CAT AGA AGT GTA GAC TTT TTT AAT      1658
Thr Ile Pro Phe Phe Thr Trp Thr His Arg Ser Val Asp Phe Phe Asn
490                 495                 500                 505

ACA ATT GAT GCT GAA AAG ATT ACT CAA CTT CCA GTA GTG AAA GCA TAT      1706
Thr Ile Asp Ala Glu Lys Ile Thr Gln Leu Pro Val Val Lys Ala Tyr
                510                 515                 520

GCC TTG TCT TCA GGT GCT TCC ATT ATT GAA GGT CCA GGA TTC ACA GGA      1754
Ala Leu Ser Ser Gly Ala Ser Ile Ile Glu Gly Pro Gly Phe Thr Gly
            525                 530                 535

GGA AAT TTA CTA TTC CTA AAA GAA TCT AGT AAT TCA ATT GCT AAA TTT      1802
Gly Asn Leu Leu Phe Leu Lys Glu Ser Ser Asn Ser Ile Ala Lys Phe
        540                 545                 550

AAA GTT ACA TTA AAT TCA GCA GCC TTG TTA CAA CGA TAT CGT GTA AGA      1850
Lys Val Thr Leu Asn Ser Ala Ala Leu Leu Gln Arg Tyr Arg Val Arg
    555                 560                 565

ATA CGC TAT GCT TCT ACC ACT AAC TTA CGA CTT TTT GTG CAA AAT TCA      1898
Ile Arg Tyr Ala Ser Thr Thr Asn Leu Arg Leu Phe Val Gln Asn Ser
570                 575                 580                 585

AAC AAT GAT TTT ATT GTC ATC TAC ATT AAT AAA ACT ATG AAT ATA GAT      1946
Asn Asn Asp Phe Ile Val Ile Tyr Ile Asn Lys Thr Met Asn Ile Asp
                590                 595                 600

GAT GAT TTA ACA TAT CAA ACA TTT GAT CTC GCA ACT ACT AAT TCT AAT      1994
Asp Asp Leu Thr Tyr Gln Thr Phe Asp Leu Ala Thr Thr Asn Ser Asn
            605                 610                 615

ATG GGG TTC TCG GGT GAT ACG AAT GAA CTT ATA ATA GGA GCA GAA TCT      2042
Met Gly Phe Ser Gly Asp Thr Asn Glu Leu Ile Ile Gly Ala Glu Ser
        620                 625                 630

TTC GTT TCT AAT GAA AAA ATC TAT ATA GAT AAG ATA GAA TTT ATC CCA      2090
Phe Val Ser Asn Glu Lys Ile Tyr Ile Asp Lys Ile Glu Phe Ile Pro
    635                 640                 645

GTA CAA TTG TAAGGAGATT TTGAAATGTA GGGCGATGGT CAAAATGAAA              2139
Val Gln Leu
650

GAATAGGAAG GTGAATTTTG ATGGTTAGGA AAGATTCTTT TAAGAAAAGC AACATGGAAA    2199

AGTATACAGT ACAAATATTA GAAATAAAAT TTATTAACAC AGGGGAAGAT GGTAAACCAG    2259
```

WSDOCID: <WO 9213954A1>

AACCGTATGG TTATATTGAC TTTTATTATC AACCTGCTCC TAACCTGAGA GAAGAAAAAG 2319

TAAGAATTTG GGAAGAGAAA AATAGTAGCT CTCCACCTTC AATAGAAGTT ATTACGGGGC 2379

TAACTTTTAA TATCATGGCT ACTTCACTTA GCCGATTATG TTTTGAAGGT T 2430

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 652 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Asn Pro Asn Asn Arg Ser Glu His Asp Thr Ile Lys Val Thr Pro
1 5 10 15

Asn Ser Glu Leu Pro Thr Asn His Asn Gln Tyr Pro Leu Ala Asp Asn
20 25 30

Pro Asn Ser Thr Leu Glu Glu Leu Asn Tyr Lys Glu Phe Leu Arg Met
35 40 45

Thr Glu Asp Ser Ser Thr Glu Val Leu Asp Asn Ser Thr Val Lys Asp
50 55 60

Ala Val Gly Thr Gly Ile Ser Val Val Gly Gln Ile Leu Gly Val Val
65 70 75 80

Gly Val Pro Phe Ala Gly Ala Leu Thr Ser Phe Tyr Gln Ser Phe Leu
85 90 95

Asp Thr Ile Trp Pro Ser Asp Ala Asp Pro Trp Lys Ala Phe Met Ala
100 105 110

Gln Val Glu Val Leu Ile Asp Lys Lys Ile Glu Glu Tyr Ala Lys Ser
115 120 125

Lys Ala Leu Ala Glu Leu Gln Gly Leu Gln Asn Asn Phe Glu Asp Tyr
130 135 140

Val Asn Ala Leu Asn Ser Trp Lys Lys Thr Pro Leu Ser Leu Arg Ser
145 150 155 160

Lys Arg Ser Gln Asp Arg Ile Arg Glu Leu Phe Ser Gln Ala Glu Ser
165 170 175

His Phe Arg Asn Ser Met Pro Ser Phe Ala Val Ser Lys Phe Glu Val
180 185 190

Leu Phe Leu Pro Thr Tyr Ala Gln Ala Ala Asn Thr His Leu Leu Leu
195 200 205

Leu Lys Asp Ala Gln Val Phe Gly Glu Glu Trp Gly Tyr Ser Ser Glu
210 215 220

DOCID: <WO 9213954A1>

Asp Val Ala Glu Phe Tyr His Arg Gln Leu Lys Leu Thr Gln Gln Tyr
225 230 235 240

Thr Asp His Cys Val Asn Trp Tyr Asn Val Gly Leu Asn Gly Leu Arg
245 250 255

Gly Ser Thr Tyr Asp Ala Trp Val Lys Phe Asn Arg Phe Arg Arg Glu
260 265 270

Met Thr Leu Thr Val Leu Asp Leu Ile Val Leu Phe Pro Phe Tyr Asp
275 280 285

Val Arg Leu Tyr Ser Lys Gly Val Lys Thr Glu Leu Thr Arg Asp Ile
290 295 300

Phe Thr Asp Pro Ile Phe Ser Leu Asn Thr Leu Gln Glu Tyr Gly Pro
305 310 315 320

Thr Phe Leu Ser Ile Glu Asn Ser Ile Arg Lys Pro His Leu Phe Asp
325 330 335

Tyr Leu Gln Gly Ile Glu Phe His Thr Arg Leu Gln Pro Gly Tyr Ser
340 345 350

Gly Lys Asp Ser Phe Asn Tyr Trp Ser Gly Asn Tyr Val Glu Thr Arg
355 360 365

Pro Ser Ile Gly Ser Ser Lys Thr Ile Thr Ser Pro Phe Tyr Gly Asp
370 375 380

Lys Ser Thr Glu Pro Val Gln Lys Leu Ser Phe Asp Gly Gln Lys Val
385 390 395 400

Tyr Arg Thr Ile Ala Asn Thr Asp Val Ala Ala Trp Pro Asn Gly Lys
405 410 415

Ile Tyr Phe Gly Val Thr Lys Val Asp Phe Ser Gln Tyr Asp Asp Gln
420 425 430

Lys Asn Glu Thr Ser Thr Gln Thr Tyr Asp Ser Lys Arg Asn Asn Gly
435 440 445

His Val Gly Ala Gln Asp Ser Ile Asp Gln Leu Pro Pro Glu Thr Thr
450 455 460

Asp Glu Pro Leu Glu Lys Ala Tyr Ser His Gln Leu Asn Tyr Ala Glu
465 470 475 480

Cys Phe Leu Met Gln Asp Arg Arg Gly Thr Ile Pro Phe Phe Thr Trp
485 490 495

Thr His Arg Ser Val Asp Phe Phe Asn Thr Ile Asp Ala Glu Lys Ile
500 505 510

Thr Gln Leu Pro Val Val Lys Ala Tyr Ala Leu Ser Ser Gly Ala Ser
515 520 525

Ile Ile Glu Gly Pro Gly Phe Thr Gly Gly Asn Leu Leu Phe Leu Lys
530 535 540

```
Glu Ser Ser Asn Ser Ile Ala Lys Phe Lys Val Thr Leu Asn Ser Ala
545                 550                 555                 560

Ala Leu Leu Gln Arg Tyr Arg Val Arg Ile Arg Tyr Ala Ser Thr Thr
                565                 570                 575

Asn Leu Arg Leu Phe Val Gln Asn Ser Asn Asn Asp Phe Ile Val Ile
            580                 585                 590

Tyr Ile Asn Lys Thr Met Asn Ile Asp Asp Asp Leu Thr Tyr Gln Thr
        595                 600                 605

Phe Asp Leu Ala Thr Thr Asn Ser Asn Met Gly Phe Ser Gly Asp Thr
    610                 615                 620

Asn Glu Leu Ile Ile Gly Ala Glu Ser Phe Val Ser Asn Glu Lys Ile
625                 630                 635                 640

Tyr Ile Asp Lys Ile Glu Phe Ile Pro Val Gln Leu
                645                 650
```

ANNEX M3

International Application No: PCT/ /

MICROORGANISMS See Attachment

Optional Sheet in connection with the microorganism referred to on page ____, line ____ of the description [1]

A. IDENTIFICATION OF DEPOSIT [1]

Further deposits are identified on an additional sheet ☒ [2]

Name of depositary institution [3]

American Research Culture Collection (NRRL)

Address of depositary institution (including postal code and country) [4]

1815 N. University Street
Peoria, Illinois 61604 United States of America

Date of deposit [5]	Accession Number [6]
See Attachment	See Attachment

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC).

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

(January 1985)

ISDOCID: <WO 9213954A1>

ATTACHMENT TO FORM PCT/RO/134

CONTINUATION OF "MICROORGANISM" BOX:

page 10, lines 3-11
page 26, lines 21-22
page 28, lines 10-12
page 42, lines 22-24
page 47, lines 26-27

CONTINUATION OF IDENTIFICATION OF DEPOSIT BOX A:

The following microorganisms were deposited in the depository institution listed in Box A on the dates listed below:

Bacterial Strain	NRRL Acession No.	Date of Deposit
B. thuringiensis EG2158	B-18213	29 April 1987
B. thuringiensis HD73-26	B-18508	12 June 1989
B. thuringiensis EG2838	B-18603	8 February 1990
B. thuringiensis EG5144	B-18655	22 May 1990
B. thuringiensis EG7237	B-18736	17 October 1990
B. thuringiensis EG5145	B-18920	21 November 1991
E. coli EG7236	B-18662	6 June 1990

)OCID: <WO 9213954A1>

CLAIMS

WE CLAIM:

1. A purified and isolated *cryIIIC(b)* gene characterized in that its nucleotide base sequence encodes the amino acid sequence illustrated in Figure 1 (SEQ ID NO:2).

2. A purified and isolated *cryIIIC(b)* gene according to claim 1 further characterized in that the gene has a coding region extending from nucleotide bases 144 to 2099 in the nucleotide base sequence illustrated in Figure 1 (SEQ ID NO:1).

3. A recombinant plasmid containing the gene of claim 1 or claim 2.

4. A coleopteran-toxic protein produced by the gene of claim 1 or claim 2.

5. A biologically pure culture of a bacterium transformed with the recombinant plasmid of claim 3.

6. The bacterium of claim 5 further characterized in that the bacterium is *Bacillus thuringiensis*.

7. The *Bacillus thuringiensis* bacterium of claim 6 deposited with the NRRL with accession number NRRL B-18736.

8. An insecticide composition characterized in that the composition comprises the protein of claim 4 and an agriculturally acceptable carrier.

9. An insecticide composition characterized in that the composition comprises the bacterium of claim 5, a coleopteran-toxic protein produced by such bacterium, and an agriculturally acceptable carrier.

10. A plant characterized in that the plant is transformed with the gene of claim 1 or claim 2.

11. The *cryIIIC(b)* gene of claim 2 further characterized in that the gene or a portion thereof is labelled for use as a hybridization probe.

12. A biologically pure culture of a *Bacillus thuringiensis* bacterium deposited with the NRRL with accession number NRRL B-18655.

13. A coleopteran-toxic protein characteristic of that made by the *Bacillus thuringiensis* bacterium of claim 12 and having the amino acid sequence illustrated in Figure 1 (SEQ ID NO:2).

14. An insecticide composition characterized in that the composition comprises the coleopteran-toxic protein of claim 13, in combination with an agriculturally acceptable carrier.

15. The insecticide composition of claim 14 further characterized in that the coleopteran-toxic protein is associated with a *Bacillus thuringiensis* bacterium which has produced such protein.

16. A method of controlling coleopteran insects characterized by applying to a host plant for such insects an insecticidally effective amount of the coleopteran-toxic protein of claim 4.

17. The method of claim 16 further characterized in that the coleopteran-toxic protein is associated with a *Bacillus thuringiensis* bacterium which has produced such protein.

SDOCID: <WO 9213954A1>

18. The method according to claim 16 further characterized in that the insects are selected from the group consisting of corn rootworms, Mexican bean beetles and Japanese beetle larvae.

19. A method of controlling coleopteran insects characterized by applying to a host plant for such insects an insecticidally effective amount of the coleopteran-toxic protein of claim 13.

20. The method of claim 19 further characterized in that the coleopteran-toxic protein is associated with a *Bacillus thuringiensis* bacterium which has produced such protein.

21. The method of claim 19 further characterized in that the insects are selected from the group consisting of corn rootworms, Mexican bean beetles and Japanese beetle larvae.

22. A biologically pure culture of a *Bacillus thuringiensis* bacterium deposited with the NRRL with accession number NRRL B-18920.

23. An insecticide composition characterized in that the composition comprises the coleopteran-toxic protein obtainable from the *Bacillus thuringiensis* bacterium of claim 12 or claim 22, in combination with an agriculturally acceptable carrier.

FIGURE 1-1

cryIIIC(b)

```
        10         20         30         40         50         60
CCATATACAACTTATCAGGAAGGGGGGGATGCACAAAGAAGAAAAGAATAAGAAGTGAAT

        70         80         90        100        110        120
GTTTATAATGTTCAATAGTTTTATGGGAAGGCATTTTATCAGGTAGAAAGTTATGTATTA

       130        140        150        160        170        180
TGATAAGAATGGGAGGAAGAAAAATGAATCCAAACAATCGAAGTGAACATGATACGATAA
           RBS          MetAsnProAsnAsnArgSerGluHisAspThrIleL

       190        200        210        220        230        240
AGGTTACACCTAACAGTGAATTGCCAACTAACCATAATCAATATCCTTTAGCTGACAATC
ysValThrProAsnSerGluLeuProThrAsnHisAsnGlnTyrProLeuAlaAspAsnP

       250        260        270        280        290        300
CAAATTCGACACTAGAAGAATTAAATTATAAAGAATTTTTAAGAATGACTGAAGACAGTT
roAsnSerThrLeuGluGluLeuAsnTyrLysGluPheLeuArgMetThrGluAspSerS

       310        320        330        340        350        360
CTACGGAAGTGCTAGACAACTCTACAGTAAAAGATGCAGTTGGGACAGGAATTTCTGTTG
erThrGluValLeuAspAsnSerThrValLysAspAlaValGlyThrGlyIleSerValV

       370        380        390        400        410        420
TAGGGCAGATTTTAGGTGTTGTAGGAGTTCCATTTGCTGGGGCACTCACTTCATTTTATC
alGlyGlnIleLeuGlyValValGlyValProPheAlaGlyAlaLeuThrSerPheTyrG

       430        440        450        460        470        480
AATCATTTCTTGACACTATATGGCCAAGTGATGCTGACCCATGGAAGGCTTTTATGGCAC
lnSerPheLeuAspThrIleTrpProSerAspAlaAspProTrpLysAlaPheMetAlaG

       490        500        510        520        530        540
AAGTTGAAGTACTGATAGATAAGAAAATAGAGGAGTATGCTAAAAGTAAAGCTCTTGCAG
lnValGluValLeuIleAspLysLysIleGluGluTyrAlaLysSerLysAlaLeuAlaG

       550        560        570        580        590        600
AGTTACAGGGTCTTCAAAATAATTTCGAAGATTATGTTAATGCGTTAAATTCCTGGAAGA
luLeuGlnGlyLeuGlnAsnAsnPheGluAspTyrValAsnAlaLeuAsnSerTrpLysL

       610        620        630        640        650        660
AAACACCTTTAAGTTTGCGAAGTAAAAGAAGCCAAGATCGAATAAGGGAACTTTTTTCTC
ysThrProLeuSerLeuArgSerLysArgSerGlnAspArgIleArgGluLeuPheSerG

       670        680        690        700        710        720
AAGCAGAAAGTCATTTTCGTAATTCCATGCCGTCATTTGCAGTTTCCAAATTCGAAGTGC
lnAlaGluSerHisPheArgAsnSerMetProSerPheAlaValSerLysPheGluValL
```

DOCID: <WO 9213954A1>

FIGURE 1-2

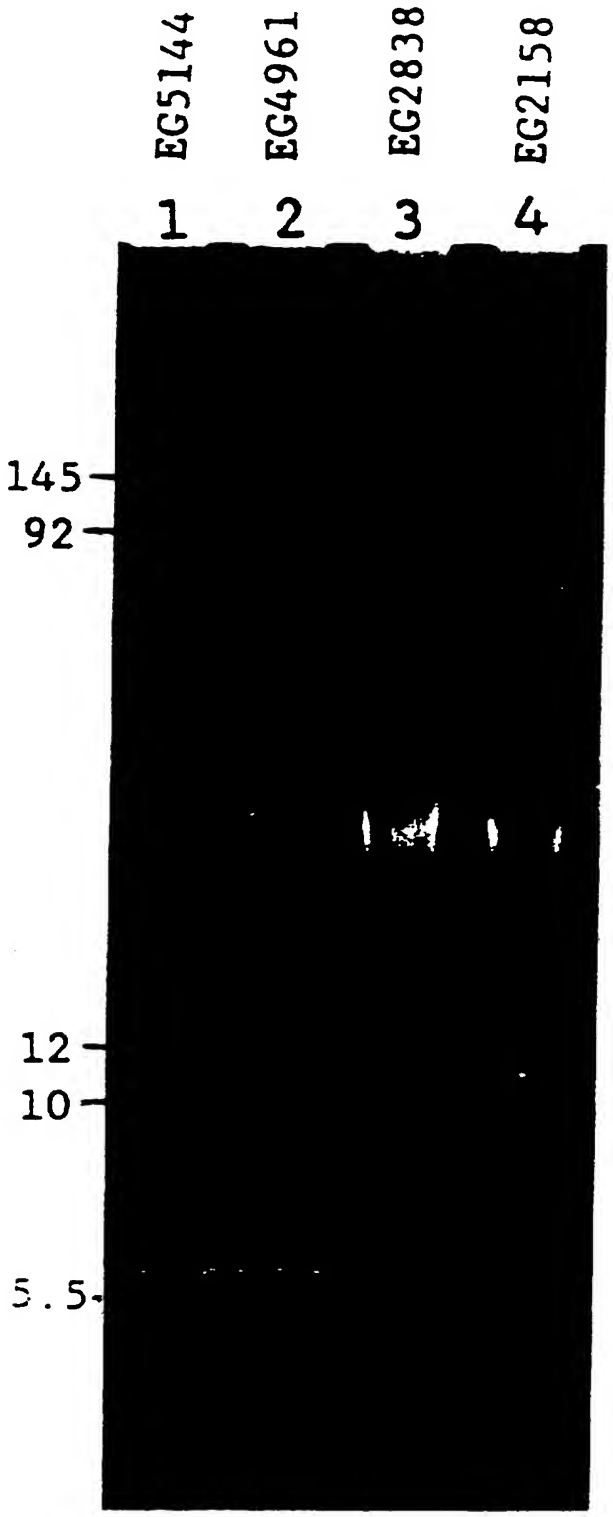
```
       850        860        870        880        890        900
AATTAAAACTTACGCAACAATACACTGACCATTGTGTCAATTGGTATAATGTTGGATTAA
lnLeuLysLeuThrGlnGlnTyrThrAspHisCysValAsnTrpTyrAsnValGlyLeuA

       910        920        930        940        950        960
ATGGTTTAAGAGGTTCAACTTATGATGCATGGGTCAAATTTAACCGTTTTCGCAGAGAAA
snGlyLeuArgGlySerThrTyrAspAlaTrpValLysPheAsnArgPheArgArgGluM

       970        980        990       1000       1010       1020
TGACTTTAACTGTATTAGATCTAATTGTACTTTTCCCATTTTATGATGTTCGGTTATACT
etThrLeuThrValLeuAspLeuIleValLeuPheProPheTyrAspValArgLeuTyrS

      1030       1040       1050       1060       1070       1080
CAAAAGGTGTTAAAACAGAACTAACAAGAGACATTTTTACGGATCCAATTTTTTCACTCA
erLysGlyValLysThrGluLeuThrArgAspIlePheThrAspProIlePheSerLeuA

      1090       1100       1110       1120       1130       1140
ATACTCTTCAGGAGTATGGACCAACTTTTTTGAGTATAGAAAACTCTATTCGAAAACCTC
snThrLeuGlnGluTyrGlyProThrPheLeuSerIleGluAsnSerIleArgLysProH

      1150       1160       1170       1180       1190       1200
ATTTATTTGATTATTTACAGGGTATTGAATTTCATACGCGTCTTCAACCTGGTTACTCTG
isLeuPheAspTyrLeuGlnGlyIleGluPheHisThrArgLeuGlnProGlyTyrSerG

      1210       1220       1230       1240       1250       1260
GGAAAGATTCTTTCAATTATTGGTCTGGTAATTATGTAGAAACTAGACCTAGTATAGGAT
lyLysAspSerPheAsnTyrTrpSerGlyAsnTyrValGluThrArgProSerIleGlyS

      1270       1280       1290       1300       1310       1320
CTAGTAAGACAATTACTTCCCCATTTTATGGAGATAAATCTACTGAACCTGTACAAAAGT
erSerLysThrIleThrSerProPheTyrGlyAspLysSerThrGluProValGlnLysL

 HindIII
    /  1330       1340       1350       1360       1370       1380
TAAGCTTTGATGGACAAAAAGTTTATCGAACTATAGCTAATACAGACGTAGCGGCTTGGC
euSerPheAspGlyGlnLysValTyrArgThrIleAlaAsnThrAspValAlaAlaTrpP

      1390       1400       1410       1420       1430       1440
CGAATGGCAAGATATATTTTGGTGTTACGAAAGTTGATTTTAGTCAATATGATGATCAAA
roAsnGlyLysIleTyrPheGlyValThrLysValAspPheSerGlnTyrAspAspGlnL

      1450       1460       1470       1480       1490       1500
AAAATGAAACTAGTACACAAACATATGATTCAAAAAGAAACAATGGCCATGTAGGTGCAC
ysAsnGluThrSerThrGlnThrTyrAspSerLysArgAsnAsnGlyHisValGlyAlaG

      1510       1520       1530       1540       1550       1560
AGGATTCTATTGACCAATTACCACCAGAAACAACAGATGAACCACTTGAAAAAGCATATA
lnAspSerIleAspGlnLeuProProGluThrThrAspGluProLeuGluLysAlaTyrS
```

ISDOCID: <WO 9213954A1>

FIGURE 1-3

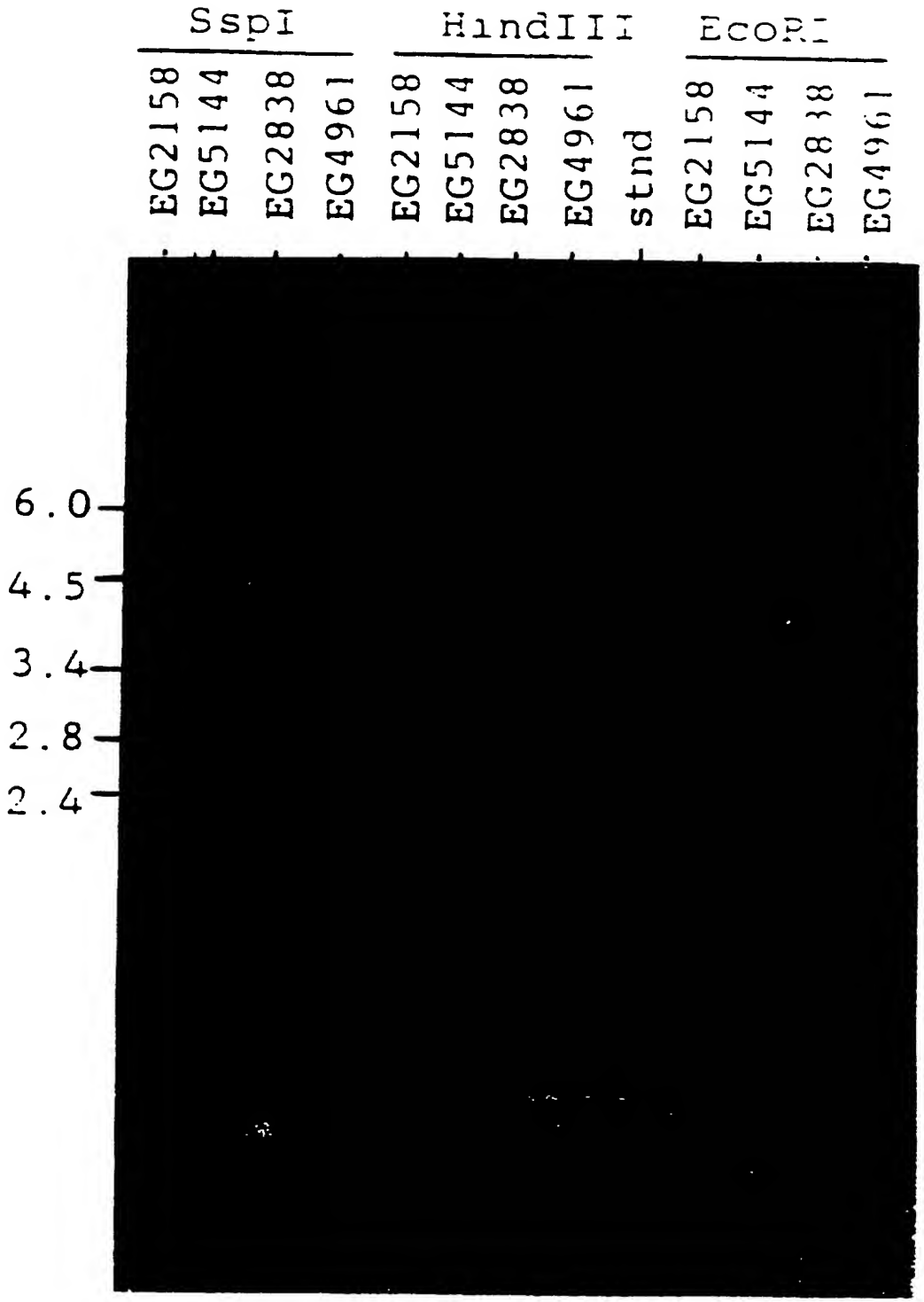
```
      1690       1700       1710       1720       1730       1740
CTCAACTTCCAGTAGTGAAAGCATATGCCTTGTCTTCAGGTGCTTCCATTATTGAAGGTC
hrGlnLeuProValValLysAlaTyrAlaLeuSerSerGlyAlaSerIleIleGluGlyP

      1750       1760       1770       1780       1790       1800
CAGGATTCACAGGAGGAAATTTACTATTCCTAAAAGAATCTAGTAATTCAATTGCTAAAT
roGlyPheThrGlyGlyAsnLeuLeuPheLeuLysGluSerSerAsnSerIleAlaLysP

      1810       1820       1830       1840       1850       1860
TTAAAGTTACATTAAATTCAGCAGCCTTGTTACAACGATATCGTGTAAGAATACGCTATG
heLysValThrLeuAsnSerAlaAlaLeuLeuGlnArgTyrArgValArgIleArgTyrA

      1870       1880       1890       1900       1910       1920
CTTCTACCACTAACTTACGACTTTTTGTGCAAAATTCAAACAATGATTTTATTGTCATCT
laSerThrThrAsnLeuArgLeuPheValGlnAsnSerAsnAsnAspPheIleValIleT

      1930       1940       1950       1960       1970       1980
ACATTAATAAAACTATGAATATAGATGATGATTTAACATATCAAACATTTGATCTCGCAA
yrIleAsnLysThrMetAsnIleAspAspAspLeuThrTyrGlnThrPheAspLeuAlaT

      1990       2000       2010       2020       2030       2040
CTACTAATTCTAATATGGGGTTCTCGGGTGATACGAATGAACTTATAATAGGAGCAGAAT
hrThrAsnSerAsnMetGlyPheSerGlyAspThrAsnGluLeuIleIleGlyAlaGluS

      2050       2060       2070       2080       2090       2100
CTTTCGTTTCTAATGAAAAAATCTATATAGATAAGATAGAATTTATCCCAGTACAATTGT
erPheValSerAsnGluLysIleTyrIleAspLysIleGluPheIleProValGlnLeuE

      2110       2120       2130       2140       2150       2160
AAGGAGATTTTGAAATGTAGGGCGATGGTCAAAATGAAAGAATAGGAAGGTGAATTTTGA
nd

      2170       2180       2190       2200       2210       2220
TGGTTAGGAAAGATTCTTTTAAGAAAAGCAACATGGAAAAGTATACAGTACAAATATTAG

      2230       2240       2250       2260       2270       2280
AAATAAAATTTATTAACACAGGGGAAGATGGTAAACCAGAACCGTATGGTTATATTGACT

      2290       2300       2310       2320       2330       2340
TTTATTATCAACCTGCTCCTAACCTGAGAGAAGAAAAAGTAAGAATTTGGGAAGAGAAAA

      2350       2360       2370       2380       2390       2400
ATAGTAGCTCTCCACCTTCAATAGAAGTTATTACGGGGCTAACTTTTAATATCATGGCTA

      2410       2420       2430
CTTCACTTAGCCGATTATGTTTTGAAGGTT
```

FIGURE 2



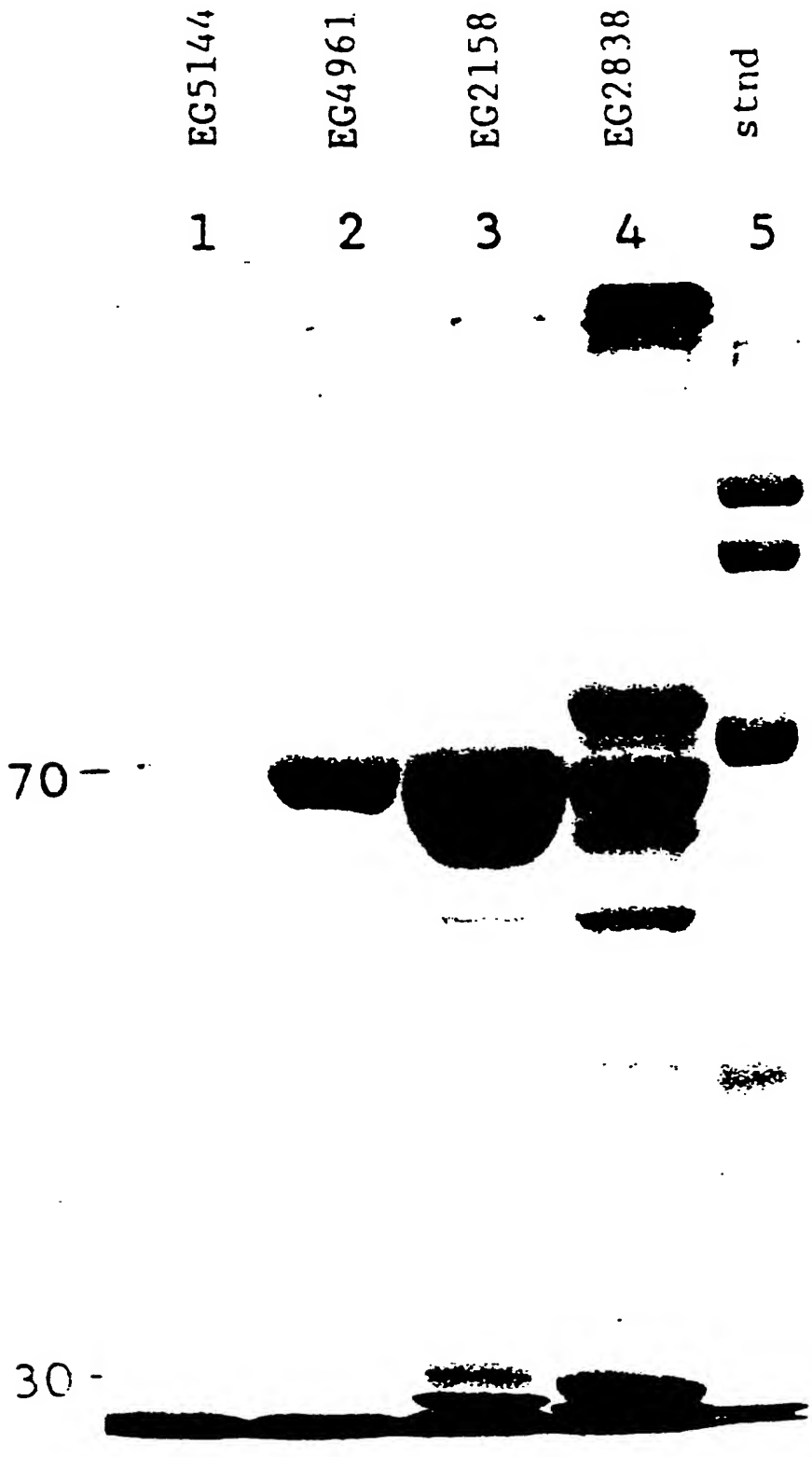
NSDOCID: <WO 9213954A1>

FIGURE 3



DOCID: <WO 9213954A1>

FIGURE 4



ISDOCID: <WO 9213954A1>

FIGURE 5

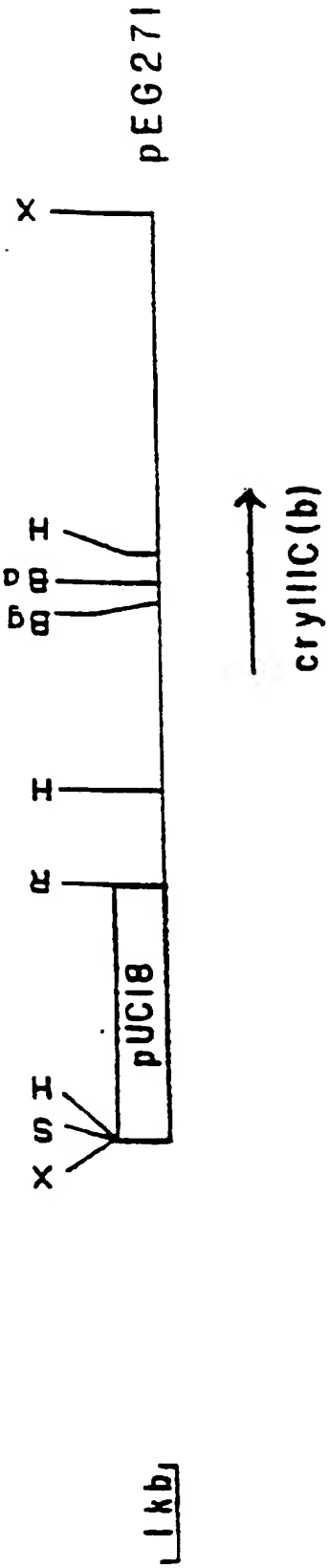
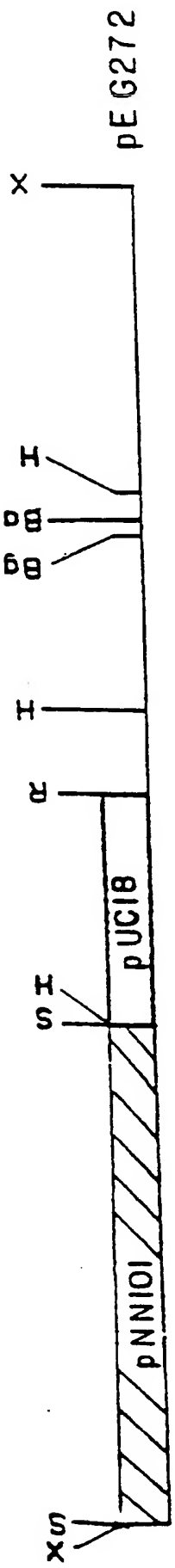
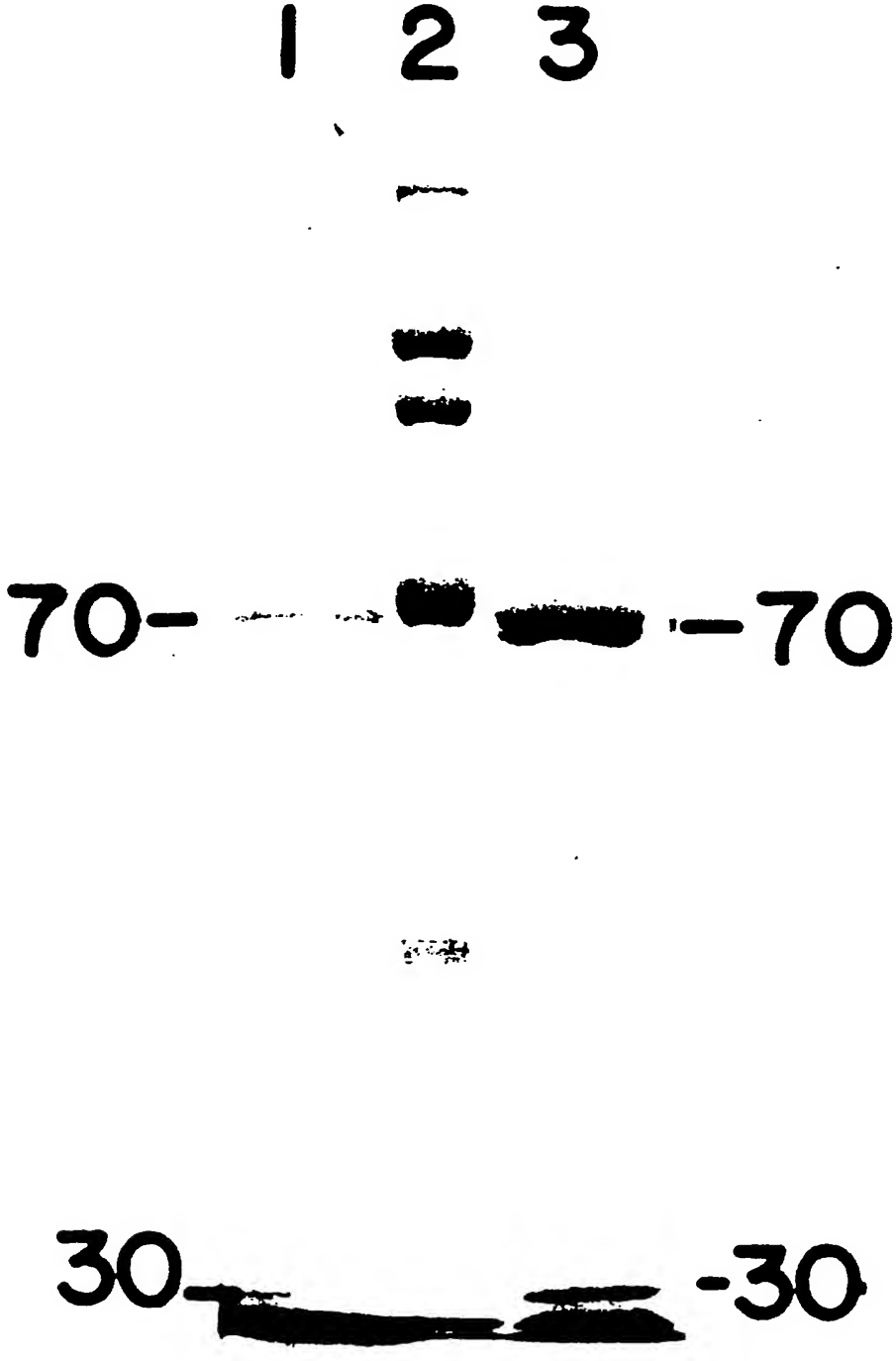


FIGURE 6



DOCID: <WO 9213954A1>

FIGURE 7



INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/00040

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/32; A01N63/02; C12Q1/68; C12N1/21
C07K13/00; //(C12N1/21; C12R1:07; 1:19)

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; A01N ; C12N ; C12R

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	EP,A,0 382 990 (PLANT GENETIC SYSTEMS, N.V.) 22 August 1990 cited in the application see the whole document, and especially figure 1 ---	1-23
A	WO,A,8 808 880 (ECOGEN, INCORPORATED) 17 November 1988 see the whole document & US,A,5 024 837 (ECOGEN, INC.) 18 June 1991 cited in the application ---	
A	EP,A,0 328 383 (MYCOGEN CORPORATION) 16 August 1989 cited in the application see the whole document ---	1-23

-/—

* Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13 APRIL 1992	21. 04. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	ANDRES S.M.

Form PCT/ISA/210 (second sheet) (January 1985)

DOCID: <WO 9213954A1>

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	WO,A,9 116 433 (PLANT GENETIC SYSTEMS, N.V.) 31 October 1991 see page 20; example 3 see page 38; figure 2	1-23
P,X	WO,A,9 114 778 (ECOGEN, INC.) 3 October 1991 see the whole document	1-23

ISDOCID: <WO 9213954A1>

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9200040 SA 56074

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 13/04/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0382990	22-08-90	AU-A-	5047690	05-09-90
		WO-A-	9009445	23-08-90
		EP-A-	0458819	04-12-91
WO-A-8808880	17-11-88	US-A-	5024837	18-06-91
		AU-B-	617110	21-11-91
		AU-A-	1782388	06-12-88
		EP-A-	0359771	28-03-90
		JP-T-	2501439	24-05-90
US-A-5024837	18-06-91	AU-B-	617110	21-11-91
		AU-A-	1782388	06-12-88
		EP-A-	0359771	28-03-90
		JP-T-	2501439	24-05-90
		WO-A-	8808880	17-11-88
EP-A-0328383	16-08-89	US-A-	4999192	12-03-91
		JP-A-	2131414	21-05-90
WO-A-9116433	31-10-91	AU-A-	7752691	11-11-91
WO-A-9114778	03-10-91	AU-A-	7555291	21-10-91

EPO FORM P079

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

DOCID: <WO 9213954A1>